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Acta haemat. 56: 1-6 (1976)

The Distribution of B and T Lymphocytes in the Peripheral Blood of Patients with Hodgkin's Disease

AMIRA MANY, MIRIAM BENIAMINOV, ESTHER ROSENTHAL, ESTHER AGHAI and BRACHA RAMOT

The Chaim Sheba Medical Center, Tel-Hashomer, and Tel-Aviv University Sackler School of Medicine, Tel-Aviv

Key Words. Hodgkin's disease · B lymphocytes · T lymphocytes

Abstract. The distribution of thymus-dependent lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) was studied in 74 patients with Hodgkin's disease and 33 normal controls. A T cell deficit was found in untreated patients as well as in long-term survivors in remission. Therapy slightly enhanced the T cell depletion in Hodgkin's disease patients. Concomitant with this finding was a slight increase of B cells.

Abnormalities in cell-mediated immune reactions in patients with Hodgkin's disease have been well documented [1, 2, 5, 11–13, 19, 20]. Conflicting observations are recorded, however, as to the distribution of thymus-dependent lymphocytes (T cells) and bone-marrow-derived lymphocytes (B cells) in the peripheral blood of such patients [3, 6–10, 15].

This study presents the number of T and B cells in the peripheral blood of 74 patients with Hodgkin's disease at different stages of the disease, treated and untreated.

Materials and Methods

The test group consisted of 74 patients representing all clinical stages and histologic cell types of Hodgkin's disease; 25 of them untreated. The control group consisted of 33 healthy laboratory personnel and medical students, matched for age and sex with the group of patients. The differentiation between B and T cells was based upon the presence of demonstrable surface immunoglobulins on B cells, and the ability to form spontaneous rosettes with sheep red blood cells (E rosettes) of T cells.

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Table I. Distribution of T and B cells in 74 patients with Hodgkin's disease and 33 normal controls

Groups	B cells/ mm	T cells mm
Normal controls compared to patients		
Normal controls (33)	576 ± 224	1,950 ± 489
Hodgkin's disease (74)	709 ± 423	860 ± 396
p	<0.05	<0.0005
Distribution in different histological types of untreated patients		
Lymphocyte predominant (4)	789 ± 201	1,150 ± 353
Nodular sclerosis (15)	704 ± 375	978 ± 397
Mixed cellularity (4)	950 ± 603	1,400 ± 141
Lymphocyte depletion (2)	215 ± 49	723 ± 3
Patients in long-term remission compared to normal controls		
Patients in long remission (19)	835 ± 471	806 ± 316
Normal controls (33)	576 ± 224	1,950 ± 489
p	<0.005	<0.0005

Groups	B cells/ mm^3	T cells/ mm^3
Normal controls compared to untreated patients		
Normal controls (33)	576 ± 224	1,950 ± 489
Untreated patients (25)	761 ± 414	985 ± 445
p	<0.025	<0.0005
Distribution in different clinical stages of untreated patients		
I and II (15)	845 ± 471	1,075 ± 373
III and IV (10)	526 ± 258	727 ± 276
p	<0.025	<0.05
Patients in long-term remission compared to untreated patients		
Patients in long remission (19)	835 ± 471	806 ± 316
Untreated patients (25)	761 ± 414	985 ± 445
p	<0.33	<0.1

Number of subjects in parentheses. Results are expressed as mean ± SD

Patients in remission without maintenance therapy for 2–15 years were compared to normal controls on the one hand and to untreated patients on the other. No significant differences were found between remission and untreated patients while remission and normal controls showed differences.

lymphocytes, 10–30% monocytes and a few granulocytes. For demonstration of membrane immunoglobulins, 1×10⁶ cells were incubated for 30 min at 0 °C with FITC conjugated goat antibodies to human IgG IgA and IgM (Meloy) After 3 washings with PBS, the cells were examined under a Zeiss fluoromicroscope [18]. Then the fluorescent lymphocytes, B cells, were counted and their percentage calculated

While enumerating the B cells, the monocytes were excluded morphologically by the phase-contrast microscope This was possible after finding a good correlation between the phase-contrast microscopy on one hand and α-naphtyl-acetate esterase and peroxidase staining as well as EA rosettes, according to SHEVACH *et al* [16], on the other

The number of T cells was determined by the E rosette test as described by STJERNSWARD *et al.* [17].

The total number of T and B lymphocytes per cubic millimeter were calculated from the white blood cells and differential counts performed on each blood sample.

Student's t test was used to assess the statistical significance of the differences observed.

Results

Table I presents the number of B and T cells per cubic millimeter in normal controls and all the patients with Hodgkin's disease. These patients show an increase of B cells as compared to the normal controls. The most striking difference between the two groups is seen in the markedly lower number of T cells recorded in the patients with Hodgkin's disease.

The relation of these results to therapy was determined by comparing 25 untreated patients to normal controls The untreated patients show an increase of B cells and a highly significant decrease of T cells The number of T cells in this group is somewhat higher than that found in the entire group of patients, showing that therapy had some effect.

The distribution of B and T cells in the four histologic types of Hodgkin's disease (Rye classification) are summarised in table I The number of patients in three of the groups is too small to draw any conclusion however the paucity of lymphocytes in the lymphocyte-depleted patients with Hodgkin's disease is noteworthy especially the severe deficit in B cells.

Significant differences were seen between non treated patients in clinical stages I II and those staged III–IV Patients staged III–IV had a normal B cell count, but their T cell deficit was greater than that of patients staged I–II

Table I. Distribution of T and B cells in 74 patients with Hodgkin's disease and 33 normal controls

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Patients in remission without maintenance therapy for 2–15 years were compared to normal controls on the one hand and to untreated patients on the other. No significant differences were found between remission and untreated patients while remission and normal controls showed differences.

Discussion

The results of the study clearly demonstrate a significant deficit in T cells in patients with Hodgkin's disease. This finding is in agreement with the documented depressed cell mediated immunity in these patients. This decrease in number of T cells was found both in untreated patients and in those who had completed therapy many years earlier.

It seems, therefore, that the decrease of T cells is a fundamental feature of Hodgkin's disease, and therapy, mainly irradiation, enhances it. Our observation could support ORDER and HELLMAN's [14] hypothesis that Hodgkin's disease evolves as a response of competent T cells to virus-infected T cells, which then leads to the development of neoplastic reticulum cells. If this course of events is correct, depletion of circulating T cells is expected. No correlation could be found between the histological type of the disease and the extent of T cell deficit in view of the small number of patients examined in three of the groups.

A correlation appears to exist between the clinical stage of the disease and the number of T cells, the T cell deficit being more pronounced in the more advanced stages of the disease, namely III and IV. This observation corresponds with LEVY and KAPLAN's [12] findings of impaired T lymphocyte function in all stages of Hodgkin's disease, starting from I_A and increasing in the more advanced stages. This impairment was also found in patients in long term remission.

It is not clear whether the depletion in T cells or E rosette forming cells, in patients with Hodgkin's disease, is really quantitative, or whether it is a matter of functionally impaired T cells. The fact that all patients have normal or high numbers of B cells in spite of a lymphopenia supports the first possibility. On the other hand, we have recently shown that a short administration of levamisole, a non-specific immunostimulant, increased the number of E rosettes in patients with Hodgkin's disease. This effect was also shown *in vitro* by incubating lymphocytes of patients with Hodgkin's disease with levamisole [submitted for publication]. These results support a qualitative defect of T cells in Hodgkin's disease.

An unexpected finding was the increase of B-cells in Hodgkin's disease patients. This appears not to be related to irradiation as suggested by FALLETTA *et al.* [7], since it was seen in untreated patients as well as in those in long-term remission. This increase in B cells might stem from laboratory techniques in which the cell suspension is contaminated by monocytes. An attempt was made to avoid this by differentiating the im-

munoglobulin-bearing monocytes from the immunoglobulin-bearing B lymphocytes morphologically as mentioned in detail before. We have no explanation for the increase in B cells, which might be one of the heterogeneous cell reactions to Hodgkin's disease.

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La réserve de granulocytes médullaires chez des patients soumis à l'hémodialyse périodique

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Key Words. Ethiocholanolone test · Granulocyte reserve · Haemodialysis · Uremia

Abstract. We have studied the granulocyte reserve of bone marrow (ethiocholanolone test), the morphological aspects and the mitotic index of the granulopoietic line in 10 patients undergoing long-term haemodialysis. The granulocyte reserve was reduced in 7 cases. This phenomenon is probably due to the granulocyte loss which occurs in dialysis. The granulopoietic cells showed cytoplasmatic vacuolization and lysis of chromatin. The mitotic index was at the upper limits or above.

Nous avons observé que chez les patients soumis à une hémodialyse extracorporelle, il se produisait une perte chronique de leucocytes sédimentés sur les membranes dialysantes; nous avons calculé que cette perte équivalait à 10×10^{9} cellules [1]. Parallèlement à ce phénomène, dans la première phase de la dialyse, il se manifeste une importante neutropénie transitoire par séquestration des leucocytes surtout au niveau pulmonaire [3, 4]. Les recherches que nous avons faites nous permettent de supposer que ce phénomène est dû aux modifications subies par les premiers contingents de leucocytes au contact des membranes dialysantes [2]. La disparition du phénomène pourrait dépendre de la diminution rapide de la nocivité des membranes. Le rétablissement du nombre des leucocytes dépend de la libération des leucocytes séquestrés et, en partie, d'une réaction médullaire. Il semble certain que la neutropénie précoce aussi comporte la perte d'un contingent de leucocytes [4].

Comme ces phénomènes se manifestent dans toutes les dialyses, il est possible qu'à la longue ils puissent déterminer une déficience de la lignée granulopoïétique. Il nous a donc semblé intéressant d'étudier chez

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un groupe de patients soumis depuis longtemps à l'hémodialyse périodique pour urémie, la réserve des granulocytes médullaires, les aspects morphologiques et l'index mitotique de la lignée granulopoïétique.

Matériel et méthodes

La réserve de granulocytes médullaires a été étudiée chez 10 patients soumis à l'hémodialyse périodique pour urémie (2 dialyses par semaine de 11 h chacune avec le rein de Kill) en utilisant le test à l'éthiocholanolone (1 mg/kg par voie intramusculaire). Les leucocytes ont été comptés au microscope avant l'injection et 12, 15, 18 h après l'injection. En même temps, des frottis de sang ont été faits pour la détermination de la formule leucocytaire. Nous avons considéré normale la réserve granulocytaire lorsque l'augmentation de granulocytes était égale ou supérieure à 2600/μl.

En outre, chez chaque patient une biopsie médullaire a été pratiquée pour l'étude morphologique et l'évaluation de l'index mitotique des cellules granulopoïétiques.

Résultats

Parmi les cas examinés, 2 patients seulement présentent une réponse granulocytaire normale; chez l'un la réponse est aux limites inférieures de la norme (cas 6); dans les autres les variations des granulocytes sont minimales ou absentes (tab. I).

L'étude de la moelle montre que la lignée granulopoïétique est bien représentée du point de vue quantitatif. On observe des phénomènes de vacuolisation du cytoplasme et de lyse de la chromatine dans un certain nombre d'éléments. L'index mitotique des cellules granulopoïétiques se situe constamment aux limites supérieures de la norme ou même au-delà (tab. I).

Discussion

Les résultats démontrent que dans la grande majorité des patients examinés il existe une réduction nette de la réserve de granulocytes médullaires. On peut attribuer ce phénomène à la perte chronique des granulocytes qui se produit dans toute dialyse. Il est toutefois possible qu'à cette cause s'associent d'autres facteurs, toxiques, carentiels; on pourrait attribuer les altérations morphologiques décrites à ces derniers facteurs. L'élévation de l'index mitotique des cellules granulopoïétiques peut être considérée comme l'expression de la sollicitation de la prolifération.

Tableau I. Nombre de granulocytes avant et après éthiocholanolone et index mitotique des cellules granulopoïétiques

Cas n°	Age	Sexe	Mois de dialyse	Diagnostic	Granulocytes/µl		Index mitotique /‰
					avant	après	
1	61	M	30	NC	5 450	5 750	7
2	35	M	37	NC	2 800	5 500	8
3	4[illegible]	M	49	PNC	2 450	5 000	11
4	58	F	23	NC	5 100	5 200	8
5	43	M	[illegible]3	NC	5 000	5 100	10
6	46	F	6	NC	2 450	4 200	6
7	47	M	38	NC	5 600	5 800	11
8	48	M	38	NC	4 700	5 100	9
9	21	F	62	NC	4 800	4 800	12
10	51	F	36	RPC	4 200	4 300	7
Valeurs normales				augmentation	5	2 600	6–8

Pour les valeurs obtenues après l'injection, nous donnons la valeur la plus élevée. NC = Néphrite chronique PNC = pyélonéphrite chronique RPC = rein polycystique.

Résumé

Nous avons étudié la réserve de granulocytes médullaires (test à l'éthiocholanolone), la morphologie et l'index mitotique de la lignée granulopoïétique chez 10 patients soumis depuis longtemps à l'hémodialyse extracorporelle avec le rein de Kiil. Chez 7 d'entre eux, la réserve de granulocytes médullaire est réduite. C phénomène semble être lié à la perte de granulocytes lors de chaque dialyse. Les éléments de la lignée granulopoïétique montrent des phénomènes de vacuolisation cytoplasmatique et de lyse de la chromatine. L'index mitotique des cellules granulopoïétiques se situe à la limite supérieure de la norme ou même au-delà.

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un groupe de patients soumis depuis longtemps à l hémodialyse périodique pour urémie, la réserve des granulocytes médullaires les aspects morphologiques et l index mitotique de la lignée granulopoïétique.

Matériel et méthodes

La réserve de granulocytes médullaires a été étudiée chez 10 patients soumis à l'hémodialyse périodique pour urémie (2 dialyses par semaine de 11 h chacune avec le rein de Kill) en utilisant le test à l éthiocholanolone (1 mg/kg par voie intramusculaire) Les leucocytes ont été comptés au microscope avant l'injection et 12, 15 18 h après l'injection. En même temps, des frottis de sang ont été faits pour la détermination de la formule leucocytaire Nous avons considéré normale la réserve granulocytaire lorsque l augmentation de granulocytes était égale ou supérieure à 600 / l

En outre chez chaque patient une biopsie médullaire a été pratiquée pour l étude morphologique et l évaluation de l index mitotique des cellules granulopoïétiques.

Résultats

Parmi les cas examinés, 2 patients seulement présentent une réponse granulocytaire normale chez l un la réponse est aux limites inférieures de la norme (cas 6) dans les autres les variations des granulocytes sont minimales ou absentes (tab I)

L étude de la mœlle montre que la lignée granulopoïétique est bien représentée du point de vue quantitatif On observe des phénomènes de vacuolisation du cytoplasme et de lyse de la chromatine dans un certain nombre d éléments. L index mitotique des cellules granulopoïétiques se situe constamment aux limites supérieures de la norme ou même au-delà (tab I)

Discussion

Les résultats démontrent que dans la grande majorité des patients examinés il existe une réduction nette de la réserve de granulocytes médullaires. On peut attribuer ce phénomène à la perte chronique des granulocytes qui se produit dans toute dialyse Il est toutefois possible qu à cette cause s associent d autres facteurs toxiques carentiels on pourrait attribuer les altérations morphologiques décrites à ces derniers facteurs. L élévation de l index mitotique des cellules granulopoïétiques peut être considérée comme l expression de la sollicitation de la prolifération

Tableau I. Nombre de granulocytes avant et après éthiocholanolone et index mitotique des cellules granulopoïétiques

Cas n°	Age	Sexe	Mois de dialyse	Diagnostic	Granulocytes/µl avant	Granulocytes/µl après	Index mitotique ‰
1	61	M	30	NC	5 450	5 750	7
2	35	M	37	NC	800	5 500	8
3	47	M	49	PNC	2 450	5 000	11
4	58	F	[illegible]	NC	5 100	5 200	8
5	43	M	[illegible]	NC	5 000	5 100	10
6	46	F	6	NC	450	4 200	6
7	47	M	38	NC	5 600	5 800	11
8	48	M	38	NC	4 700	5 100	9
9	21	F	62	NC	4 800	4 800	12
10	51	F	36	RPC	4 200	4 300	7
Valeurs normales				augmentation	[illegible]	2 600	6–8

Pour les valeurs obtenues après l'injection, nous donnons la valeur la plus élevée. NC = Néphrite chronique PNC = pyélonéphrite chronique RPC = rein polycystique.

Résumé

Nous avons étudié la réserve de granulocytes médullaires (test à l'éthiocholanolone), la morphologie et l'index mitotique de la lignée granulopoïétique chez 10 patients soumis depuis longtemps à l'hémodialyse extracorporelle avec le rein de Kiil. Chez 7 d'entre eux, la réserve de granulocytes médullaire est réduite. Ce phénomène semble être lié à la perte de granulocytes lors de chaque dialyse. Les éléments de la lignée granulopoïétique montrent des phénomènes de vacuolisation cytoplasmatique et de lyse de la chromatine. L'index mitotique des cellules granulopoïétiques se situe à la limite supérieure de la norme ou même au-delà.

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En outre, chez chaque patient une biopsie médullaire a été pratiquée pour l'étude morphologique et l évaluation de l'index mitotique des cellules granulopoïétiques.

Résultats

Parmi les cas examinés 2 patients seulement présentent une réponse granulocytaire normale chez l un la réponse est aux limites inférieures de la norme (cas 6) dans les autres les variations des granulocytes sont minimales ou absentes (tab I)

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Les résultats démontrent que dans la grande majorité des patients examinés il existe une réduction nette de la réserve de granulocytes médullaires On peut attribuer ce phénomène à la perte chronique des granulocytes qui se produit dans toute dialyse Il est toutefois possible qu à cette cause s associent d autres facteurs toxiques, carentiels on pourrait attribuer les altérations morphologiques décrites à ces derniers facteurs.

L élévation de l'index mitotique des cellules granulopoïétiques peut être considérée comme l expression de la sollicitation de la prolifération

Table I

Patient No	Sex	Age	Clinical diagnosis
1	M	73 y	pernicious anaemia
	M	63 y	pernicious anaemia
3	M	68 y	pernicious anaemia
4	F	77 y	pernicious anaemia
5	F	65 y	pernicious anaemia
6	M	60 y	folic acid deficiency
7	M	m	folic acid deficiency
8	F	77 y	folic acid deficiency
9	F	14 y	folic acid-dependent megaloblastic anaemia

rectly into cold 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.2. Marrow particles were then separated and fixed in fresh 2% glutaraldehyde for h at 4 °C, rinsed in 0.1 M phosphate buffer with sucrose, post-fixed in osmium tetroxide, dehydrated in increasing series f acetone and embedded in Vestopal-W. The sections were cut on Reichert ultramicrotome OMU III, mounted on carbon-coated cooper grids, stained with uranyl acetate and lead citrate and examined in JEOL 100-B electron microscope at 60 kV. I order to achieve easier identification of ferritin particles, some sections were examined fter staining with uranyl acetate 'en bloc' without further contrast ith lead citrate. In each case, at least 100 erythroblasts were examined.

Results

In three (No. 1 5 and 8) out of nine patients, we found multiple cytoplasmic connections between adjacent erythroblasts (fig. 1). These junctions have a zipper like appearance. The erythroblasts presenting junctions were usually at the same stage of maturation. Less frequently contacts between two erythroblasts of different degree of maturation were observed. No rhopheocytosis could be seen at the connecting points, but it was detected at other sites of the erythroblastic surface (fig. 1)

At high magnifications, the connecting membranes were strictly parallel and were separated by a constant space of approximately 20 nm. The material situated in the connecting space between erythroblasts, responsible for the zipper-like appearance, was ferritin as could be demonstrated in sections only stained en bloc' with uranyl acetate (fig. 2)

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Cytoplasmic Connections between Erythroblasts in Megaloblastic Anaemia

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Key Words. Dyserythropoiesis · Electron microscopy · Erythroblastic connections · Erythroblastic synartesis · Megaloblastic anaemia

Abstract. In three out of nine cases of megaloblastic anaemia, multiple cytoplasmic connections between adjacent erythroblasts were found. The similarity and the difference with respect to so-called 'erythroblastic synartesis' are discussed.

With the term 'erythroblastic synartesis' BRETON-GORIUS *et al.* [1] and FLANDRIN *et al.* [2] have described a case of refractory anaemia presenting at the ultrastructural level peculiar intererythroblastic junctions. According to FRISCH and LEWIS [4] similar connections to those of erythroblastic synartesis can be seen in patients with megaloblastic anaemia, leukaemia and hereditary spherocytosis. These authors feel that the phenomenon is just another nonspecific feature of dyserythropoiesis. But FLANDRIN and BRETON-GORIUS [3] think that the cellular junctions referred to by FRISCH and LEWIS [4] were not clearly demonstrated at the appropriate magnifications, and that they might be different to those described as 'erythroblastic synartesis'.

The previous observations aroused our interest in order to analyze the relationship between adjacent erythroblasts in megaloblastic anaemia.

Material and Methods

The bone marrow of 9 patients suffering megaloblastic anaemia was examined (table I). The following method was used: aspirated sternal marrow was placed di

Discussion

The cytoplasmic connections of the so-called erythroblastic synartesis were septate-like junctions between strictly parallel membranes of connecting erythroblasts [1, 2]. In a comparative study of a case of sideroblastic refractory anaemia, FLANDRIN *et al.* [2] found intererythroblastic contacts which appeared at lower magnifications similar to those described in erythroblastic synartesis, but looked different at high magnifications, in two points: (a) the membranes were not strictly parallel, and (b) instead of septate-like junctions, particles of ferritin were found in the connecting space between erythroblasts.

The cytoplasmic connections we have observed in 3 out of 9 cases of megaloblastic anaemia are at low magnifications also very similar to those described as erythroblastic synartesis. At high magnifications, the similarity persists as the parallelism of the connecting membranes is concerned. The *chief difference is the presence of ferritin particles instead of* the septate-like material.

The significance of this type of connections is unknown. It is possible, as FRISCH and LEWIS [4] feel, that they are just another expression of dyserythropoiesis. Our findings are in agreement with this statement, but it would be advisable to analyze the erythroblastic contacts in other disorders of red cell series, showing the dyserythropoietic features or not.

Acknowledgments. We are grateful to Mrs. MARÍA JOSÉ BERA for her technical assistance and to Mrs. BEGOÑA RAMÍREZ for her secretarial work.

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Fig 1 Multiple cytoplasmic connections between two adjacent erythroblasts (arrows). The double arrow points to a vesicle of rhopheocytosis. The bar corresponds to 0.75 μm × 22,500.



Fig 2 The strict parallelism of the connecting membranes is demonstrated (arrows) The very slight staining allows better identifications of ferritin particles in the space between the membranes. The bar corresponds to 0.[illegible] μm. Section stained only with uranyl acetate 'en bloc' without further contrast with lead citrate × 100 000

Discussion

The cytoplasmic connections of the so-called erythroblastic synartesis were septate like junctions between strictly parallel membranes of connecting erythroblasts [1, 2]. In a comparative study of a case of sideroblastic refractory anaemia, FLANDRIN *et al.* [2] found intererythroblastic contacts which appeared at lower magnifications similar to those described in 'erythroblastic synartesis' but looked different at high magnifications, in two points: (a) the membranes were not strictly parallel, and (b) instead of septate-like junctions, particles of ferritin were found in the connecting space between erythroblasts.

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The significance of this type of connections is unknown. It is possible, as FRISCH and LEWIS [4] feel, that they are just another expression of dyserythropoiesis. Our findings are in agreement with this statement, but it would be advisable to analyze the erythroblastic contacts in other disorders of red cell series, showing the dyserythropoietic features or not.

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Erythrocyte 2,3-Diphosphoglycerate and Adenosine-Triphosphate in Cretins Living at High Altitude

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Key Words Anemia ATP 2,3-Diphosphoglycerate Erythropoietin Hypothyroidism Hypoxia

Abstract A comparison of concentrations of 2,3-diphosphoglycerate (2,3-DPG) and adenosine-triphosphate (ATP) in the red cells of cretins and normal controls living at 3 700 m in the Nepal Himalayas has shown that 2,3-DPG and ATP levels were higher in the cretins. A negative correlation between hemoglobin and 2,3-DPG levels was found. Chronic hypoxia appears to have provided the additional stress required to differentiate the significance of thyroid hormone deficiency in producing anemia from its effect on 2,3-DPG levels. If thyroid hormone is in fact one regulator of 2,3-DPG the anemia of hypothyroidism appears to be more significant. This also suggest that the anemia of hypothyroidism is, at least in part, 'pathologic' as opposed to adaptive

Previous studies have demonstrated that thyroid hormones can stimulate erythropoiesis in animals [1 2] and that thyroidectomy is associated with decreased red cell production [3] The mechanism by which thyroid hormone affects erythropoiesis has been considered to be mediated by erythropoietin [4] the elaboration of which is generally assumed to be commensurate with tissue O_2 requirements *[5] This concept is supported by* studies of hypopituitarism in which red cell 2,3-diphosphoglycerate (2,3-DPG) was found to be normal despite a marked decrease in red cell mass [6] The conclusion was that the anemia of hypopituitarism was adaptive rather than pathologic The possibility that a similar adaptive mechanism explains the anemia of hypothyroidism has been confounded by reports that [1] the hemoglobin (Hb) of hypothyroid subjects has increased O_2 affinity [7] and a decrease in O_2 release [8] and [2] thyroid hormones have a direct effect on erythropoiesis in rats [9] The present study was

undertaken in a unique field situation to attempt a functional definition of the anemia of hypothyroidism.

Subjects and Methods

Four subjects, 2 cretins and 2 controls, were studied in the village of Lang-Tang, altitude 3,700 m, in northern Nepal. All were of pure Tibetan ancestry. One cretin was female. The control subjects had no goiters and a normal physical examination. The aspects of the cretins were classic, no thyroid gland was palpable and they had the typical sluggish movement and low mentally associated with this disease. It should be mentioned that cretinism, deaf-mutism, and myxedema are not rare in this area, and goiters are commonly encountered. The cause of this is primarily the extremely low iodine content of soil and water [10].

Heparinized and nonanticoagulated blood specimens were collected in vacutainers simultaneously from all 4 subjects and processed concurrently at high altitude. Microhematocrits, Hb levels (cyanmethemoglobin method), and reticulocyte counts were performed using standard techniques. For 2,3-DPG, adenosine-triphosphate (ATP) and lactate assays, 2 ml of 6% perchloric acid were added to 4 ml of whole blood for deproteinization. The mixture was then centrifuged and 4 ml of supernatant were neutralized with 0.55 ml of 1 N triethanolamine containing 3 N KOH. Plasma samples were similarly processed. Specimens were then frozen, returned from the village to low altitude by air the following day and stored at −20 °C until received 10 days later in the United States. Assays of red cell 2,3-DPG, ATP, whole blood lactate, serum creatinine, free thyroxin, and inorganic phosphorus were performed at the US Naval Blood Research Laboratory, Chelsea, Mass.

Results

Table I lists the results of the various determinations. The 2,3-DPG and ATP values of the cretins were higher than the controls. Inorganic phosphorus levels were similar in all subjects, indicating they did not produce the different 2,3-DPG values [11, 12]. Lactate levels were similar, suggesting that aerobic metabolism was normal. Peripheral blood smears, reticulocyte counts, and mean corpuscular hemoglobin concentrations (MCHC) were normal in all. Thyroxin values of the cretins were extremely low. It should be noted that 2,3-DPG and ATP values were lower than sea level values for normal Westerners. This may reflect a technical defect in preparation of the perchloric acid extracts in a field situation, although there is some evidence that persons of Tibetan ancestry may be unique in their control of Hb levels [13, 14]. In either event all specimens

Table I Comparison of various red cell and serum determinations on cretins and normal persons living at 3 700 m

	Sex	Age, years	Hb, g %	MCHC,	2,3-DPG[1] µM/g Hb	ATP[1] µM/g Hb	Whole blood lactate,[1] µM/ml	Serum creatinine, mg/100 ml	Free thyroxin,[1] mg/100 ml	Serum inorganic phosphorus, mg/100 ml
Cretin No. 1	F	27	11 5	32.4	12.07	3 91	0.94	0.8	0.45	4.7
Cretin No	M	34	13 9	3_3	11 29	3 64	0.84	0.6	0.35	4 7
Control No. 1	M	5?	14 4	33 7	10.29	3 48	0.62	0.8	ND	5 4
Control No. _	M	36	16.6	33.3	10 06	3.34	1 14	0.8	ND	5.9

Normal values _3-DPG 12 ± 1_ µM/g Hb ATP 4.5 ± 0 4 µM/g Hb lactate less than 1 0 µM/ml whole blood on fresh specimens, free thyroxin, 1.2-2.2 mg/100 ml.

ND = Not done because of insufficient serum clinically euthyroid

were collected and processed simultaneously and identically and either comparative or absolute values should be adequate for interpretation

Discussion

In the present study an increase in 2,3 DPG and ATP among cretins over controls living at 3 700 m was found. On the other hand, the Hb levels of the cretins were lower than the normals, being below 2 SDM for males and females found in a study of an unselected 20% of the village population [13] Thus, a negative correlation between Hb and 2,3 DPG levels was present These data suggest that, despite previous evidence for increased O_2 affinity in hypothyroidism [7 8] in the stress of sustained low O_2 tension the protective mechanism provided by 2,3-DPG against anemia remains intact Even though *in vitro* [15] and *in vivo* [16] studies have suggested that increased thyroid hormone can elevate 2,3-DPG perhaps by stimulating diphosphoglycerate mutase, it is possible that thyroid hormone alone is relatively unimportant in regulating 2,3 DPG in the hypothyroid state

MALGOR *et al* [9] have recently shown that thyroid hormones can directly stimulate erythropoiesis in nephrectomized rats. A possible corollary to this is that a severe deficiency of thyroid hormone may result in a greater degree of anemia than might be expected if only erythropoietin

mediated tissue O_2 requirements needed to be met. This could explain the higher levels of 2,3-DPG found in the cretins.

It may be argued that other causes of anemia were present in the cretins, as folate, vitamin B_{12}, and iron deficiency are frequently encountered in hypothyroid subjects [17, 18]. It was not possible to obtain adequate serum for these assays, and a deficiency state cannot be excluded. However, in a nutritional survey of this village [13], only 3 of 52 subjects (6%) had an MCHC below 32%, and none had a serum vitamin B_{12}, serum folate or red cell folate (45 subjects) in the probable subnormal range, and no abnormal Hbs or thalassemia were found [19]. It is concluded that, under the situation of chronic hypoxia of high altitude, cretinous subjects, having lower Hb levels than controls, seemed to react as many euthyroid anemic humans in having higher 2,3-DPG levels than controls. This suggests that the anemia of hypothyroidism is, at least in part, pathologic.

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Interrelations between ABO Blood Group, Plasminogen, α_1-Antitrypsin, α_2-Macroglobulin and the Platelet Count in Blood Donors

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Key Words. α_1-Antitrypsin · α_2-Macroglobulin · Blood groups · Fibrinolysis · Oral contraception · Plasminogen

Abstract. The levels of plasminogen, α_1-antitrypsin, α_2-macroglobulin and the platelet count were measured in 511 blood donors. The mean level of α_1-antitrypsin was significantly lower in men of group B compared with that of group O. No other differences between the blood groups reached statistical significance. Women had higher mean levels of α_1-antitrypsin and α_2-macroglobulin and a higher platelet count than men. The levels of plasminogen and α_1-antitrypsin were significantly higher in women using oral contraceptives compared with those who were not. The level of α_2-macroglobulin fell with age until the 60–64 year age-group in men and the 40–49 year age-group in women. A positive correlation existed between the α_1-antitrypsin and the α_2-macroglobulin level and between the platelet count and the plasminogen level.

Mourant *et al.* [21] have reviewed the evidence for the existence of an association between blood group A and thrombo-embolic disease. The haemostatic process involving platelets, the coagulation mechanism and the fibrinolytic system may be important in the genesis of thrombotic disease. The association between blood group A and thrombo-embolic disease may be mediated through factors participating in the haemostatic process. Accordingly, we have examined the interrelations between ABO blood group, age, sex, plasminogen, the antiplasmins α_2-macroglobulin and α_1-antitrypsin, and the platelet count in a group of healthy subjects.

Subjects and Methods

The subjects tested were blood donors from the City of Aberdeen, age 18-64 who had volunteered to give donations at the Regional Transfusion Centre for the preparation of cryoprecipitate. The sessions were held between 9 and 10 in the morning. All the subjects had given at least one previous donation, and were familiar with the surroundings and staff

511 donors were studied, 272 male and .39 female 6 women were ascertained to be taking oestrogen/progestogen preparations and they have been considered separately Of the remainder 204 were of group O 126 men and 78 women 220 were of group A, 118 and 102 women, and 61 were of group B, 28 male and 33 females. Group AB donors were not included in the study Of the group A donors 168 were further subdivided into A_1 (116) and A_2 (5).

The samples were obtained at the end of donations of approximately 4?0 ml of blood into Fenwal plastic bags (Travenol Laboratories Ltd Thetford), the samples being collected direct from the donor after the plastic tubing had been clipped and divided Plasma and serum were separated within 1 h of blood collection, and stored at −30 °C till assay Because of tube breakage and other laboratory accidents a few donors do not have a complete set of assay results.

Platelet counts were performed by one of the authors [M A.] by the method of OTTLE and SPRIGGS [25], within 1 h of blood collection.

Plasminogen α_1-antitrypsin and α-macroglobulin were measured as described previously [22]

Results

Blood group differences The means and SD of all five parameters and age, subdivided by ABO blood group and sex are shown in table I The mean level of α_1 antitrypsin was significantly lower for group B than group O men ($p<0.005$) No other blood group difference reached statistical significance In addition analysis of the mean values of the platelet count plasminogen, α_1-antitrypsin and α_2 macroglobulin for the donors of group A_1 compared with those of group A_2 revealed no significant differences (table II)

Differences associated with sex Table I shows that in all three blood groups the platelet count and the mean level of α_1 antitrypsin and α macroglobulin are lower in men than in women and table III shows that for the three blood groups combined, these differences are significant Table III also shows a significant increase in the levels of α antitrypsin and plasminogen in women using oral contraception compared with those who were not There was no significant difference in the platelet count

Table I. Mean values in respect of certain parameters of the fibrinolytic system, in relation to the blood ABO group of blood donors

Parameter	Blood group	Men No.	mean	SD	Women not using oral contraception No.	mean	SD
Platelet count, 10^9/l	O	114	19 1	56.4	78	232.5	56.4
	A	115	217 0	47 4	100	.39 8	59 4
	B	28	11 4	45 6	32	221.3	47.5
Plasminogen, caesin U/ml	O	119	3 71	0.76	78	3 68	0.70
	A	116	3.77	0.71	97	3 71	0 83
	B	77	3.52	0 7?	31	3.88	0 6..
α_1-Antitrypsin, mg/dl	O	123	771 7	53.5	77	287.8	62.1
	A	116	268 6	54.5	97	776.2	55 8
	B	27	43 4*	32 8	31	771 5	40 6
α_2-Macroglobulin, mg/dl	O	123	271 7	50 0	78	298 2	58.5
	A	116	264 0	49 7	97	799.2	58 2
	B	27	250 0	46.3	31	306.3	73.1
Age, years	O	121	38.1	12.2	78	40 6	12.2
	A	11	37.6	11 9	101	38.3	13.3
	B	27	36 0	9 8	32	40 0	14 2

* $p < 0.005$

Table II. Mean values for platelet count, plasminogen, α_1-antitrypsin and α_2-macroglobulin in blood donors of groups A and A_2

Parameter	Blood group	Men No	mean	SD	Women No.	mean	SD
Platelet count, 10^9/l	A	62	216 0	43 8	54	243 4	53.0
	A_2	79	217 1	48.7	20	247 4	69 4
Plasminogen, caesin U/ml	A	62	3.70	0.76	52	3.81	0 85
	A	30	3 76	0.61	20	3 60	0 66
α_1-Antitrypsin, mg/dl	A	61	269.5	49 7	52	269.2	52.2
	A_2	31	270.3	68.3	19	273.3	62.1
α_2-Macroglobulin, mg/dl	A	61	262.8	52.6	52	291.3	58.5
	A	31	.57 9	66 3	19	312.8	67.5
Age, years	A	62	35.8	11.2	54	42.0	12.3
	A	31	45.3	12.2	21	39 8	14 8

Table III Difference by sex, and by use of oral contraception in respect of certain para meters of the fibrinolytic system in blood donors

Parameter	(1) Men n = 272		(2) Woman not using oral contraception n = 213		(3) Women using oral contraception n = 26		Significance of difference p	
	mean	SD	mean	SD	mean	SD	1 vs. 2	2 vs. 3
Platelet count, × 10⁹/l	217.[illegible]	51 0	234 4	56.3	[illegible]3[illegible]5	60.	<0.001	>0.1
Plasminogen, casein U/ml	3 72	0.7[illegible]	3 72	0.72	4.14	0.87	>0.1	<0.01
α Antitrypsin, mg/dl	267 7	52.1	[illegible]79.5	54 9	361 0	91.5	<0.05	<0.001
α_2-Macroglobulin, mg/dl	67 1	50.3	299.3	59 1	3[illegible]3 0	113 2	<0 001	0.05< p<0.1
Age, years	37 7	–	39.3	–	28.4	–		

Table IV Age and sex differences in α_2-macroglobulin levels (mg/dl)

Age, years	Men			Women		
	No.	mean	SD	No.	mean	SD
18–29	78	284.3	55.5	57	3[illegible]8	69.3
30–39	69	[illegible]69 7	50.0	4	297 0	55.[illegible]
40–49	59	255 4	46.7	53	[illegible]89 0	53 5
50–59	4	43.5	39.3	39	[illegible]90 7	43 6
60–64	8	71.5	54.2	14	287 1	82.7

Age differences No correlation with age was observed for the platelet count, plasminogen and α_1 antitrypsin The relationship of age with α_2 macroglobulin is summarised in table IV The level of α_2 macroglobulin falls with increase in age in young adults of both sexes. There is a tenden cy towards a rise in men between 60 and 64 but the difference between the mean of the 50–59 years age-group and that of the 60–64 years age-group did not reach the 5% level of significance

Correlations between haemostatic factors measured Correlations between the five parameters were calculated for men and for women not ad

mitting to use of oral contraception. A positive correlation was found, both in men ($p<0.001$) and women ($p<0.02$), between α_1-antitrypsin level and α_2-macroglobulin level. There was a significant correlation both in men ($p<0.05$) and women ($p<0.05$) between platelet count and plasminogen level.

Discussion

Blood group differences. In most published series relating the ABO blood groups to the incidence of clinical occlusive vascular disease, individuals of group A have had a higher incidence than group O. Examples are to be found in both arterial and venous sides of the circulation [6, 11]: occlusive peripheral arterial disease [17], cardiac infarction [1, 5] and puerperal and oral-contraceptive-associated venous thrombo-embolism [15, 28]. Coagulation factors are presumed to be important in the development of thrombo-embolic disease and some studies have suggested an association between some of these and the ABO blood group. A small but significantly higher factor VIII level has been demonstrated in group A than in group O blood donors [16, 27]. Lower levels of antithrombin III, a finding known to be associated with pulmonary embolism, disseminated intravascular coagulation, and the use of oral contraceptives, were found in group A donors, compared with group O [9]. DINTENFASS and SHARP [6] found differences between the blood groups A and O in the rate of formation and degradation of white thrombus *in vitro* in arteriosclerotic subjects. AMARIS and MINK [2], measuring coagulation and fibrinolytic factors in a group of healthy young women, found that the women of group A had a more rapid thromboplastin generation screening test than those of group O, but the differences did not reach statistical significance. They found no other difference in the various factors measured.

In the present study no association between the factors studied and blood group have been detected apart from the reduction in α_1-antitrypsin in group B males. Although significant at the 1 in 200 level this may reflect merely the number of comparisons which have been made.

Differences associated with sex. The higher α_2-macroglobulin level in women compared with men has been noted previously [10], whereas the platelet count is usually considered to be uninfluenced by sex [30]. It is unlikely that this sex difference was due to differences in the effects of several minutes of venous compression of the arm before sampling, as a mean difference in the platelet count of only 4×10^9/l was observed in 3

men and 4 women where samples were obtained by venepuncture both before occlusion and after venous compression of the arm for the minimum and maximum times taken for blood donation

The present study confirms the striking effect of oestrogen-containing contraceptives on components of the haemostatic mechanism A rise in α_2 macroglobulin and α_1-antitrypsin during oestrogen-progestogen therapy has been well documented [13 14 18] The influence of oral contraceptives on the plasma plasminogen level is less well established Whereas PHILLIPS *et al* [26] HOWIE *et al* [14] and AMRIS and STARUP [3] observed an increase in the plasminogen level BRAKMAN and ASTRUP [4] found no difference in the mean plasminogen level between hormone treated and control women Our results support the former conclusion We were, however unable to confirm previous reports that the platelet count is increased by oestrogen progestogen preparations [19 20]

Age differences α_2 Macroglobulin levels have been found to decrease with ageing [10] but subjects over the age of 65 were not studied In the present group of blood donors the fall in the level of α_2 macroglobulin with increase in age in younger adults was also found, but there was a trend towards a rise in the small number of older men participating in the study The rise in the α_2 macroglobulin level in elderly subjects has been noted previously [12, 19]

Correlations The explanation for the positive correlation between the platelet count and the plasminogen level in healthy blood donors is obscure Platelets contain a number of components of the fibrinolytic system, in particular inhibitors of plasmin and plasminogen activation [8, 23] and plasminogen has been identified by immunological techniques in washed platelets [24] The present results might be interpreted as suggesting that platelets contribute plasminogen to the circulating blood but since plasminogen assays are performed on essentially platelet-depleted plasma the results are unlikely to be due to a direct contribution of the platelet plasminogen in the assay procedure

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Factor X Friuli Coagulation Disorder

Report of Newly Recognized Patient and Some Considerations on the Frequency of the Disease in the Friuli Area

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Key Words. Bleeding disorders · Blood coagulation · Factor X Friuli

Abstract. A newly recognized patient with the factor X Friuli coagulation disorder is presented. The propositus is a 10-year-old boy who presented excessive bleeding after tooth extraction. *This is the 11th patient with this peculiar coagulation disorder discovered in Friuli* and the 12th so far described. His parents are consanguineous and heterozygotes together with other family members. The main laboratory features, typical of the disease, were prolonged prothrombin time, prolonged partial thromboplastin time, and normal Stypven-cephalin clotting time. The incidence of the homozygotes in the Friuli area appear to be 0.000005 (11 cases in population of 2,000,000). The probable frequency of the abnormal f gene is 0.0033, whereas that of the normal gene F is 0.9967. The theoretical frequency of the heterozygote, Ff condition is 0.007, namely 7 heterozygotes every 1,000 persons. However, only about 70 ascertained heterozygotes have been discovered so far.

Ten out of the eleven patients, so far described to have the factor X Friuli coagulation disorder, were all born in a small, isolated valley of the northeastern Italian region called Friuli [2–5]. Only one patient was described outside Friuli [10]. He came from a small village of northeastern Yugoslavia and appeared to be the result of an independent mutation. However, the geographical distance from the Friuli area is so limited that a common origin may not be completely ruled out, even though we failed to track down any relationship between this case and any of the Friulian patients.

It is likely that some of the patients with alleged factor VII deficien-

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cy and a defective thromboplastin generation described in the past, might have been cases of Friuli abnormality However this remains to be proven Until this is disproven it may be assumed that the mutation responsible for the Friuli abnormality has occurred only in the isolated valley of western Friuli Because of this geographical isolation the disease has a considerable theoretical and practical interest The reasons for such an occurrence are unknown The reasons, whereby the mutation is maintained once it has manifested itself have already been dealt with by us [5 12] The original population of the valley is about 5 000 people and has remained isolated still a few years ago causing an increased rate of intermarriages. Only a few names are common in the valley indicating a common background for most of the population Under these circumstances the perpetuation of the defect may be easily understood

This is a report of a newly recognized patient with this disorder together with some considerations about the frequency of the condition in the larger Friuli area.

Case Report

The propositus is a 10-year-old child who was first seen by us in Padua in June, 1975 The parents are consanguineous (second cousins) and family history was partially positive for bleeding disorders, since the father presented excessive bleeding after tooth extractions (fig. 1) The patient was first noted to present easy bruising at the age of 2. On the contrary epistaxis has been rare. At the age of 6 the patient presented profuse bleeding after a traumatic laceration of the tip of the nose A suture had to be applied on two occasions.

A few months before he was studied in Padua, the patient underwent two tooth extractions. Bleeding was profuse in spite of nonspecific coagulant therapy The bleeding continued for several days. At that time a routine coagulation study carried out at the Maniago City Hospital showed prolonged partial thromboplastin and prothrombin times. Because of the regional origin of the patient a tentative diagnosis of factor X Friuli disorder was formulated, and the patient was sent to Padua for further evaluation. At the time of study in Padua there were no bleeding manifestations. Physical examination revealed a well-developed child with no significant findings.

Material and Methods

These have been reported in our previous papers [7 9 11] The coagulation studies in the propositus and his parent were carried out on fresh plasma drawn in Padua The factor X assays in the other family members were carried out on frozen plasma, taken to Padua immediately after drawing and separation.

■ Propositus, homozygote symptomatic

◧ or ◐ Heterozygote partially symptomatic

□ or ○ Studied, normal

◫ or ⦶ Not studied, asymptomatic, normal ?

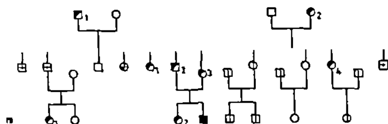


Fig. 1. Family pedigree of our propositus. Six family members, besides both parents, were found to be heterozygotes. The parents of our propositus were consanguineous.

Results

The results of the coagulation studies are summarized in table I. The prothrombin time was prolonged and corrected by normal serum and by factor VII-deficient plasma. Adsorbed normal plasma, factor X-deficient plasma and the plasma of the index patient with the Friuli abnormality failed to correct the prothrombin time (table II).

P P test, partial thromboplastin time, prothrombin consumption and thromboplastin generation test were abnormal. The substitution of the patient's serum with normal serum in the thromboplastin generation test system corrected the abnormality.

The Stypven-cephalin clotting time was normal. The factor X level was low only when tissue thromboplastin was used in the assay. On the contrary it was normal or near normal when an S-C mixture was used. All other clotting factors were within normal limits. Vascular and platelet tests were normal and there was no hyperfibrinolysis. The thromboelastogram showed a prolonged r+K, whereas ma was normal.

The cross-over electrophoresis showed that factor X Friuli has the same mobility as normal factor X. No factor X band was evident in factor X-deficient plasma (fig. 2).

Table I Coagulation study in our propositus

Test	Propositus	Normal values
Clotting time, min	1	5–9
Bleeding time, min	4	5
Clot retraction	complete in 10 h	complete after 12 h
Platelet count/μl	200,000	150,000–350,000
Prothrombin consumption, %	75	>90
TGT	34 sec in 8 min	<16 sec in 6 or 8 min
Partial thromboplastin time, sec	77.9	3 –42
Prothrombin time sec	34.5	13–14
Stypven-cephalin clotting time, sec	13.6	11 13
Thrombin time, sec	23	18–25
Factor II V VII VIII, IX, XI XII	normal	60–160%
Factor X (tissue thromboplastin), %	9	85–120
Factor X (Stypven-cephalin), %	90	85–120
TEG r mm	33	10–20
k, mm	1	6–12
ma, mm	62	50–65

Table II Prothrombin time (PT) correction studies in our propositus

Mixture (equal parts)	PT of mixture sec	PT reference plasma, sec
Propositus plasma	34.5	
Propositus plasma + plasma of index patient with Friuli abnormality	33	33.5
Propositus plasma + factor X deficient plasma	48	116
Propositus plasma + factor VII deficient plasma	15.5	55.1
Propositus plasma + normal serum	15.2	
Propositus plasma + adsorbed normal plasma	48.0	

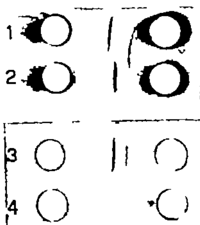


Fig 2 Cross-over electrophoresis of (1) propositus plasma (2) pooled normal plasma, (3) Dade normal plasma, and (4) classical factor X deficiency A normal major or factor X precipitate is seen in the propositus (1). No major or factor X precipitate is visible in classical factor X deficiency (4). Smaller and lighter bands are visible in all plasmas, but they may be disregarded since they represent secondary activities of the antiserum used.

Table III Factor X levels and clinical features of the heterozygotes found in the pedigree (fig. 1)

Position in family tree	Factor X level, %	Clinical features
I	58	asymptomatic so far
I	60	asymptomatic so far
II	68	excessive bleeding after tooth extraction
II	56	excessive bleeding after tooth extractions on several occasions
II_3	70	asymptomatic so far
II_4	58	excessive bleeding after tooth extraction, occasional menorrhagias
III	58	excessive bleeding after tooth extraction
III_9	58	excessive bleeding after tooth extraction

The propositus parents were found to have slightly reduced factor X levels and were considered to be heterozygotes. The same was true for other members of the family (fig. 1).

Table II Age (at the time of study), sex and main features of the 12 patients so far recognized to have the abnormal factor X (factor X Friuli) coagulation disorder

Case No.	Age	Sex	Bleeding manifestations	Comment
1	67	F	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas, menorrhagias	index patient deceased (adrenal gland hemorrhage, carcinoma of the head of the pancreas). Cases 1 2, 3 and 4 are relatives
2	40	M	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hemarthrosis	deceased (acute liver failure secondary to transfusion hepatitis)
3	46	M	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas	deceased (acute liver failure secondary to transfusion hepatitis)
4	8	M	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas	
5	13	M	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas	
6	15	M	epistaxis, bleeding from the gums bleeding after tooth extractions, posttraumatic hematomas	
7	66	F	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas, postpartum hemorrhage	not related to previous patients but coming from same valley
8	43	F	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas, bleeding after surgery postpartum hemorrhage, hematuria	

Table IV (continued)

Case No.	Age	Sex	Bleeding manifestations	Comment
9	45	M	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas	maternal cousin of case 3
10	32	F	easy bruising, bleeding after tooth extractions, bleeding after surgery	only patient so far described outside Friuli
11	1.5	M	scrotal hematoma at birth, easy bruising	not related to previous patients but coming from same valley
12	10	M	Bleeding after tooth extractions, easy bruising	present case same as for case 5

Discussion

The present case is of some interest because it had gone unrecognized in spite of our extensive studies in the valley during the past 6 years. 4 years ago we found a paternal cousin to be heterozygote but no data were gathered on the propositus or his parents. Having had no major challenge to his hemostatic mechanism he went undetected. His mild bleeding tendency did not raise any suspicion. In this regard it has to be emphasized once again that even the heterozygote population may present easy bruising or occasional bleeding after tooth extractions.

It is interesting also to note how constant the findings are in the patients with the Friuli abnormality. The propositus is the 12th patient with this peculiar disorder to be recognized so far. The prothrombin time and the partial thromboplastin time are remarkably similar in all these patients. The prothrombin time, using a rabbit brain and lung thromboplastin, has varied between 29.3 and 36.7 sec in all our patients during all these years. Partial thromboplastin times were also quite uniform since they varied only from 66.6 to 81.5. This seems a striking fact (table V). The same is true for the factor X level as determined with RVV and cephalin or with tissue thromboplastin. This seems to indicate that the phenotypic expression of the disease is quite fixed from one individual to the other. This is also true for the clinical findings, both in

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7	66	F	epistaxis, bleeding from the gums, bleeding after tooth extractions, posttraumatic hematomas, postpartum hemorrhage	not related to previous patients but coming from same valley
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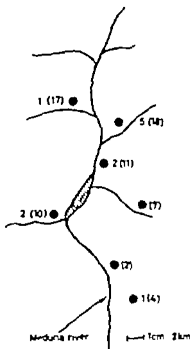


Fig. 3 Geographical distribution of homozygotes and heterozygotes in the valley. The dark dots represent the villages located in the valley. The first numbers shown near the village symbols refer to the homozygote patients. The numbers in parentheses refer to the heterozygotes.

ing that so far no patient with the same defect has been described outside Friuli with the exception of the patient from western Yugoslavia described by us a few years ago [10]

A sure diagnosis of the defect does not require sophisticated tests. It may be easily reached on the basis of three simple plasmatic screening tests, a prolonged partial thromboplastin time, a prolonged prothrombin time and a normal Stypven-cephalin clotting time. No other known coagulation disorder presents this pattern.

The topographic distribution of the eleven Friulian patients is reported in figure 3. The valley has a population of about 5,000. It is located in the western part of the northeastern Italian region called Friuli and is about 25 km long. To the north the valley is limited by a mountaneous pass which in the past was closed most of the winter time because of

Table V Main laboratory features in the 12 patients with factor X Friuli disorder so far recognized averages remarkably similar in all patients

Case No.	Prothrombin time, sec	Partial thromboplastin time, sec	Stypven-cephalin clotting time, sec
1	33 7	66.6	14
2	29.3	67 7	12
3	35.5	73 0	14 7
4	33.2	80.7	13
5	31 0	81.5	13.5
6	30.5	69 1	12.7
7	36.7	78 0	13 6
8	33.3	66.7	12
9	34 8	75.8	13
10[1]	33.5	72.5	14
11	34.5	66.6	13
12 (propositus)	34.5	77.9	13 6
Normal values	13–14	32–42	11–13

[1] Only patient so far described outside Friuli.

homozygote and heterozygote patients. The bleeding manifestations, presented by all our homozygote patients, are similar in type and always moderate in severity For example no spontaneous hemarthrosis and no cerebral hemorrhage have ever been observed (table VI) Both these bleeding manifestations have been observed on the contrary in classical factor X deficiency [1 6 17] Even the only patient, so far described outside Friuli meets these criteria [10] A fairly fixed phenotypic expression is present in the heterozygotes too They have usually factor X levels varying from 45 to 65%, and they are only occasional and mild bleeders.

The 12 homozygote cases represent already a number of patients equal to about half the cases with classical factor X deficiency Since the description of the first three cases of classical factor X deficiency in 1956–57 [1 16–18] only 22 surely additional cases have been observed and represent an extremely rare coagulation disorder [14 15] In 7 years 12 patients with the Friuli abnormality have already been gathered. This seems to indicate that the defect appears only slightly less frequent as classical factor X deficiency However the fact is really strik

the valley 689 heterozygotes should be present. Furthermore, we think that the ratio of 69 heterozygotes to 500 people is slightly biased in the sense that we have studied so far mainly relatives of homozygote patients, namely people with higher incidence for heterozygosis. In spite of this the final number (689) arrived at is definitely lower than the expected figure of 1 400.

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snow thereby increasing the isolation. It is interesting to note that in two of the small villages of the valley no homozygotes have been discovered so far. On the contrary in one small village with a population of about 400 people the impressive number of five homozygotes has been described together with 18 heterozygotes. At least half of the original population now lives outside the valley. However about 50% of this half still lives in the larger Friuli area.

Given a population of the larger Friuli area of about 2,000 000 people, the frequency of the homozygote condition appears to be about 0.000005 (11 cases out of 2,000 000 people). If that figure is duplicated to compensate for incomplete ascertainment one would have a probable upper frequency limit of 0.00001. In the past a quadruplication of the frequency was allowed to compensate for incomplete ascertainment, but we think this is unnecessary [5].

Assuming that the Hardy Weinberg formula may be applied [16] the upper limit of the abnormal gene f' frequency may be obtained from the square root of the frequency of the homozygous group

$$q = \sqrt{0.00001} = 0.0033$$

The frequency of the normal allele F may then be obtained as p = (1–q) or (1–0.0033) = 0.9967. The frequency of heterozygote may be calculated from the formula 2 pq or 2×(0.9967)×(0.0033) that is to say about 0.007. This indicates that, on theoretical grounds, one could expect to find approximately 7 heterozygotes every 1 000 people. This means that about 1 400 heterozygotes should live in the larger Friuli area. However we think this is an excessive number since only about 70 patients have been discovered so far. We doubt in fact that so many undetected heterozygote patients may still live in Friuli.

Unfortunately the confirmation or the denial of such an assumption could come only from the testing of the entire population of the valley or a great part thereof. Since this is unfeasible for practical purposes, we will have only to try to extrapolate from smaller studies. So far we have tested about 500 subjects in the valley and found 69 heterozygotes. Since the actual population of the valley at the present time is about 2,500 people by extrapolation we should find 276 additional heterozygotes for a total of 345 patients. 172 additional heterozygotes should be present among the approximately 1,250 people, living outside the valley but in the larger Friuli area. The same is true for the remaining 1,250 people living outside the larger Friuli area. In the entire population of

the valley 689 heterozygotes should be present. Furthermore, we think that the ratio of 69 heterozygotes to 500 people is slightly biased in the sense that we have studied so far mainly relatives of homozygote patients, namely people with higher incidence for heterozygosis. In spite of this the final number (689) arrived at is definitely lower than the expected figure of 1,400.

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Fetal Erythropoiesis and Dyserythropoiesis in Juvenile Chronic Myeloid Leukaemia[1]

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Key Words. Dyserythropoiesis · Fetal erythropoiesis · Juvenile myeloid leukaemia · Leukaemia · Red cell enzymes

Abstract. Haemoglobin fractions and 16 enzymatic activities of red cells of a patient with juvenile chronic myeloid leukaemia are compared to normal, to comparably reticulocyte-rich, non-neonatal and to fetal red cells. The activities of hexokinase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, monophosphoglycerate mutase, enolase and glucose-6-phosphate dehydrogenase are significantly increased in fetal red cells beyond the activities of cell populations with comparable reticulocytosis. The activities of these enzymes are also increased in the patient's erythrocytes. Together with a haemoglobin F concentration of 54% and a concentration of haemoglobin Bart's of 1% these variations reflect the fetal nature of the red cells. Simultaneously signs of dyserythropoiesis are found in the red cells of the patient: a very high activity of hexokinase and a low pyruvate kinase activity.

In childhood, chronic myeloid leukaemia differs from the adult type by the early manifestation of anaemia and thrombocytopenia, only a minor degree of leukocytosis, and the absence of the Philadelphia chromosome [7]. In addition, similarities to fetal erythrocytes have been observed in red cells of this disorder, e.g. haemoglobin F values comprising 30–70% of the total haemoglobin, the γ-chains of haemoglobin F showing a glycine to alanine ratio in γ_{136} as seen in newborn infants and a leftward displacement of the oxygen dissociation curve [10]. Other changes, which are similar to fetal erythropoiesis are reduced levels of haemoglobin A_2 [19] and an increased activity of glucose-6-phosphate dehydrogenase [5].

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preleukaemic states were excluded. The reticulocyte count of this group (n = 4) ranged from 9.0 to 17.0% with a mean of 13.4%.

Fetal erythrocytes (group 3). The red cells of this group were obtained from 6 premature babies with gestational ages of 24 weeks on the average (range 23–25 weeks). Birth weights were 665 g on the average (range 500–800 g). Blood for determination of enzymatic activities was obtained within 1 h after the death of these infants. The mean survival time was 29 h (from 17 to 37 h). None had received a blood transfusion. The reticulocyte count ranged from 8.4 to 20.0% with a mean of 14.2%. The mean haemoglobin value was 14.2 g/dl (11.6–16.0 g/dl).

Results

During the course of the illness the haemoglobin F concentration rose to 53.9% (table I). The last examination of the stroma-free red cell haemolysate of the patient on cellulose-acetate strips at pH 8.4 revealed reduced haemoglobin A_2 and no detectable carboanhydrase. Additionally a small distinct band could be seen migrating faster than haemoglobin A_1 in the region of haemoglobin Bart's. The concentration of this fraction was 1% of the total haemoglobin. Electrophoresis in starch gel at pH 8.6 and 7.0 confirmed the assumption that this fraction consisted of haemoglobin Bart's.

The activities of 16 enzymes of the patient measured in red cell haemolysates are shown in table II. Compared to the values of normal adults most enzymatic activities of the patient were markedly elevated. Only the activities of 2,3-diphosphoglycerate mutase and pyruvate kinase were within the normal range. Comparison of the patient's enzymatic activities with those of non-neonatal subjects with reticulocytosis showed that the increase of some enzymatic activities could not be explained alone by the young cell population in the patient's blood. These enzymes were hexokinase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, monophosphoglyceromutase, enolase and glucose-6-phosphate dehydrogenase.

Table II also shows the enzymatic activities of fetal red cells. The elevated reticulocyte count of fetal blood only partly accounts for the increased activities of all enzymes. Triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, monophosphoglyceromutase, 2,3-diphosphoglycerate mutase, enolase and glucose-6-phosphate dehydrogenase, however revealed activities which were higher than those of blood with comparable reticulocytosis. For the enzymes monophosphoglyceromutase, 2,3-diphosphoglycerate mutase, enolase and glucose-6-

In a patient with juvenile chronic myeloid leukaemia we found further up to now not described characteristics of the erythrocytes indicating a reversion to fetal erythropoiesis.

Material and Methods

Case Report. W. K. (born March 11, 1973) appeared healthy up to the age of 16 months. He then developed diarrhoea, unexplained fever and weight loss. Clinical examination revealed marked dystrophy, pallor, enlargement of liver and spleen and abdominal swelling. The inguinal lymph nodes were moderately enlarged. Bone marrow showed a predominance of myeloid cells with a shift to the left. There were very few megakaryocytes. Biopsy of a lymph node revealed the pattern of unspecific lymphadenitis. 3 months later the child was readmitted to another hospital. There was no significant change in his condition, but in addition, widespread purpura and petechiae of the skin were seen. The mucous membranes were bleeding and haematuria was observed. The results of two haematological examinations are shown in table I. Coagulation studies revealed prolonged bleeding time and reduced prothrombin time. Myeloid hyperplasia was found in the bone marrow. The child did not receive cytostatic agents. After the death at the age of 20 months, histological examination confirmed the diagnosis of myeloid leukaemia. Haemoglobin electrophoresis of parental blood enabled thalassaemia to be excluded.

Haemoglobin analysis. Quantitative haemoglobin F determination was performed by the method of BETKE *et al.* [3]. Haemoglobin was analyzed by electrophoresis on cellulose acetate strips, with densitometric quantitation of the single fractions; the method of WEIMNGER and ALEBOUYE was used [20]. Vertical starch gel electrophoresis was performed using the method described by LEHMANN and HUNTSMAN [9]. Tris-EDTA borate buffer was used.

Enzyme assays. The details of determination of enzymatic activities for hexokinase, glucosephosphate isomerase, phosphofructokinase, fructosediphosphate aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase, 2,3-diphosphoglycerate mutase, pyruvate kinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductases are described elsewhere [16]. In the assay of pyruvate kinase the concentration of phosphoenol pyruvate was 2 mM. The activity of enolase was determined by the method of BÜCHER [4], the activity of monophosphoglyceromutase by that of SUTHERLAND *et al.* [12].

Determination of enzymatic activities was performed at 37 °C. One IU is the quantity of enzyme which converts 1 µmol of the substrate in 1 min/g haemoglobin.

Blood of normal individuals (group 1). This group consisted of subjects serving as normal controls for the determination of erythrocytic enzymatic activities of patients with haemolytic syndromes. All were clinically well, none had anaemia or reticulocytosis. The data of this group were obtained from assays of 46 subjects.

Reticulocyte-rich blood (group 2). In this group were included older children and adults with reticulocytosis comparable to that observed in fetal blood. The etiologies of the chronic haemolytic syndromes varied. Patients with leukaemia or

Table II. Activities of erythrocytic enzymes of normal controls (group 1), reticulocyte-rich blood (group 2) and fetal erythrocytes (group 3) compared to those of the patient enzyme activities (mean ± SD) in IU/g Hb

	Group 1 n=46	Group 2 n=24	Group 3 n=6	Patient
Reticulocytes, %	0.7	13.4	14.2	9.6
Hexokinase	1.0 ± 0.1	2.4 ± 0.9	2.5 ± 0.4	5.9
Phosphoglucomutase	4.9 ± 1.1	5.7 ± 0.9	3.9 ± 1.1	6.2
Glucosephosphate isomerase	44.7 ± 4.8	61.8 ± 10.0	64.1 ± 15.5	70.3
Phosphofructokinase	21.0 ± 4.1	31.6 ± 4.6	28.5 ± 7.8	15.6
Aldolase	7.9 ± 1.4	11.2 ± 2.4	11.4 ± 3.3	13.4
Triosephosphate isomerase	2,180 ± 234	2,643 ± 386	2,974 ± 421	3,390
Glyceraldehyde-3-phosphate dehydrogenase	233 ± 50	301 ± 60	323 ± 52	566.4
2,3-diphosphoglycerate mutase	4.8 ± 0.6	3.9 ± 0.2	4.7 ± 0.7[1]	4.2
Phosphoglycerate kinase	138 ± 23	172 ± 75	168 ± 41	182.5
Monophosphoglyceromutase	67.8 ± 10.0	79.7 ± 21.4	105 ± 8.1	129.4
Enolase	16.1 ± 2.1	22.6 ± 6.9	42.2 ± 9.1[1]	44.2
Pyruvate kinase	13.2 ± 1.9	18.0 ± 5.9	21.7 ± 7.3	14.2
Glucose-6-phosphate dehydrogenase	11.0 ± 1.6	16.5 ± 3.9	20.8 ± 7.6[2]	4.2
6-phosphogluconic dehydrogenase	9.5 ± 1.5	12.8 ± 3.0	11.7 ± 3.9	15.9
Glutathione reductase I	9.3 ± 2.1	13.5 ± 2.5	12.6 ± 3.6	19.3
Glutathione reductase II	4.6 ± 0.8	6.6 ± 1.3	7.3 ± 2.1	11.2

[1] $p<0.001$ between group 2 and 3.
[2] $p<0.01$ between group 2 and 3.

are produced in excess and so the tetramer γ (haemoglobin Bart's) is formed. In cord blood of normal newborn infants only trace amounts (0.5%) of haemoglobin Bart's are detected (6). During the normal switch from γ- to β-chain synthesis there is a competition of β- and γ-chains for available α-chains. It is known that β-chains have a greater affinity to α-chains than γ-chains (8). So small amounts of haemoglobin Bart's can be formed.

Haemoglobin Bart's in the erythrocytes of our patient with juvenile chronic myeloid leukaemia indicated that an excess of free γ-chains is present which have combined to form the tetramer γ_4. This is a further

Table I Haematological data of the patient with juvenile chronic myeloid leukemia during the course of his illness

	July 1974 (first admission)	November 1974 (before death)
Haemoglobin, g/100 ml	10.7	7.2
Red blood cell count, ×10⁶/µl	3.4	2.8
Reticulocytes, %	6.8	9.6
Platelet count, ×10³/µl	–[1]	45
White blood cell count, ×10³/µl	22.1	86.1
Myelocytes, %	1	6
Metamyelocytes, %	3	3
Juvenile neutrophils, %	8	10
Band neutrophils, %	26	27
Segmented neurophils, %	51	31
Lymphocytes, %	8	22
Monocytes, %	3	1
Haemoglobin F %	12	53.9
Haemoglobin A_2, %	–[2]	<0.5
Carboanhydrase, %	1	–[2]
Haemoglobin Bart's, %	0	0.8

[1] Not determined.
[2] Not detectable on visual examination.

phosphate dehydrogenase the difference between enzyme activities of fetal red cells and of blood with comparable reticulocytosis was significant as shown by the t test (table II) Thus, the pattern of increased enzymatic activities in the red cells of the patient was similar to that seen in fetal erythrocytes. Only hexokinase of the patient s red cells exhibited an elevated activity which was higher than in both fetal and reticulocyte-rich blood Conversely the activity of pyruvate kinase was lower in the patient s cells than in fetal cells.

Discussion

The haemoglobin of the erythrocytes of the patient consisted of about 54% haemoglobin F 45% haemoglobin A_1 and a minimal fraction (1%) of haemoglobin Bart s.

Increased amounts of haemoglobin Bart s are observed in infants with defective α-chain synthesis, e.g., α-thalassaemia. In this disorder γ-chains

tal nature of the erythrocytes in juvenile chronic myeloid leukaemia Whether the appearance of fetal markers is a basic phenomenon of a congenital disorder or only an acquired compensatory mechanism remains open. A further possibility is an abnormal proliferation of a clone of undifferentiated stem cells [19] The increase of the haemoglobin F concentration during the course of the disease could favour the idea that the reversion to fetal erythropoiesis in this disorder is only an acquired, compensatory mechanism due to reactivation of fetal genes.

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point of similarity between the erythrocytes of juvenile chronic myeloid leukaemia and those produced during fetal life.

Enzymes of fetal and cord blood erythrocytes are characterized by many quantitative and qualitative differences as compared to those of adults [11 15] Some of these pecularities are shared by the erythrocytes of subjects with juvenile chronic myeloid leukaemia. So far elevated activity of glucose-6-phosphate dehydrogenase [5] and reduced carbonic anhydrase isozymes B and C [19] have been reported in this disorder

The activities of erythrocytic enzymes of fetal cells (group 1) were increased. When compared however to cells with a similar reticulocyte count (group 2) only single enzymes exhibited much higher activities in fetal erythrocytes (triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, monophosphoglyceromutase 2,3-diphosphoglycerate mutase enolase and glucose-6-phosphate dehydrogenase) Barthelmai and Vetrella [1] compared enzyme activities between these two groups for hexokinase, glucosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, monophosphoglyceromutase, enolase, pyruvate kinase, lactate dehydrogenase, Mg^{++} ATPase and 6-phosphogluconate dehydrogenase with the higher activities in fetal cells except for 6-phosphogluconate dehydrogenase, the activity of which was lower However these activities were not compared with those of red cells with comparable reticulocytosis.

Five of the six enzymes which in fetal red cells exhibited elevated activities beyond those seen in cell populations with comparable reticulocytosis were also increased in the patient s erythrocytes. This could reflect the fetal nature of his red cells and support the hypothesis of a proliferation of undifferentiated stem cells as a pathogenic principle in juvenile chronic myeloid leukaemia [19]

The very high activity of hexokinase in the patient s red cells could not be explained – either by the reticulocytosis or by typical fetal behaviour In patients with dyserythropoiesis, e.g. disorders like leukaemia, preleukaemic states or refractory anaemia, high activities of single enzymes were observed hexokinase activity in particular was found to be unusual high in an adult who was thought to have incipient myeloblastic leukaemia [18] Likewise relatively low pyruvate kinase activity was found in dyserythropoietic states [18] Thus, the very high activity of hexokinase and the relatively low pyruvate kinase activity of the patient s red cells may be markers of dyserythropoiesis.

In this paper we have reported further data which demonstrate the fe

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Glutathionreduktasemangel mit Membrandefekt bei Hereditärer Hämolytischer Anämie

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Key Words. Enzyme erythropathy · Glutathione reductase · Hereditary hemolytic anemia · Phospholipid metabolism · Red cell enzymes · Splenectomy

Abstract. Glutathione reductase activity and phospholipid metabolism in red cell membranes were determined in a family with hereditary hemolytic anemia. A marked decrease of glutathione reductase activity and stability of reduced glutathione was found in combination with enhanced phospholipid-phosphate metabolism and decreased activity of the membrane-stabilizing enzyme lysolecithin-acyl-transferase. In all cases splenectomy beneficially influenced the hemolytic process. Family studies revealed a dominant-autosomal genetic transmission.

Pathogenese und molekularbiologischer Primärdefekt vieler hämolytischer Krankheitsbilder sind noch immer weitgehend unbekannt. Mit grosser Wahrscheinlichkeit ist die zugrunde liegende Störung in einer Veränderung der Zellmembran, ihrer physikochemischen Beschaffenheit und biologischen Funktion zu suchen. Nachstehend wird über einen Fall mit unklaren hämolytischen Schüben berichtet, bei dem die Untersuchung der Erythrozyten eine erniedrigte Aktivität der Glutathionreduktase (GR), der Acyltransferase (AcTF), eine verminderte Stabilität des reduzierten Glutathions (GSH) und einen deutlich gesteigerten Phosphatumsatz besonders der sauren Phospholipide Phosphatidylserin (PS) und Phosphatidsäure (PA) ergab.

Kasuistik

F. Andreas, geb. 17.11.1962. Seit seinem 4. Lebensjahr litt der Knabe an paroxysmalen Bauchschmerzen, die mit Übelkeit und Ikterus einhergingen. Es bestand ein deutlicher Zusammenhang der Beschwerden mit fieberhaften Infekten.

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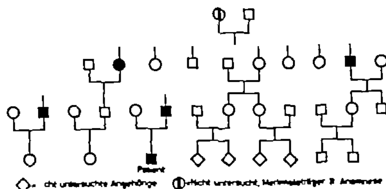


Abb. 1 Erbfolge der Merkmale GR-Mangel und Membranschädigung.

in seiner Entwicklung nicht beeinträchtigt war. Er besuchte regelmäßig die Schule, bis auf die Zeit der hämolytischen Schübe; während dieser Zeit war er 1–2 Tage bettlägerig.

Im weiteren Verlauf nahmen die Beschwerden zu. Die hämolytischen Schübe wurden häufiger und schwerer. Im Alter von 10½ Jahren gab ein schwerer hämolytischer Schub, der mit Schock und Bewusstseinsverlust einherging (Tab. I), die Indikation zur Milzexstirpation. Seit der Splenektomie ist der Patient 2 Jahre beschwerdefrei und normal belastbar. Die Untersuchungen beim Vater unseres Patienten zeigten eine identische Erkrankung, weshalb auch bei ihm die Splenektomie durchgeführt wurde. Auch er ist seither beschwerdefrei.

Material und Methoden

Neben den üblichen blutmorphologischen und klinischen Laboratoriumstests wurde eine Reihe zum Teil selbst entwickelter biochemischer Untersuchungen durchgeführt.

Erythrozytenisolierung. Da die Aktivität der meisten Erythrozytenenzyme geringer als die entsprechende in Leukozyten ist, wurde auf die exakte Abtrennung der letzteren grosser Wert gelegt. Nach der Methode von NAKAO *et al.* [14] war es möglich, die vorher dreimal mit physiologischer NaCl-Lösung bei 1000 *g* gewaschenen Erythrozyten durch Filtration über eine kleine Säule mit Sulfoäthylcellulose (Servacel SE 23) und Sephadex G-25 praktisch leukozyten- und thrombozytenfrei zu erhalten.

Retikulozytenanreicherung. Die gewaschenen und von anderen Blutkörperchen freien Erythrozyten wurden einer zweistufigen Zentrifugaltrennung bei 4 °C unterworfen [10, 12]. Von den in Polystyrolröhrchen (75 × 11 mm) 15 min bei 20 000 *g* gepackten Erythrozyten wurde das oberste und das unterste Viertel in Polyäthylenröhrchen (45 × 4 mm) 15 min bei 40 000 *g* rezentrifugiert und die jeweils leichteste

Familie (Abb 1) Schon die Urgrossmutter (väterlicherseits) hatte ein gelbliches Hautkolorit und rezidivierende Bauchschmerzen. Die Grossmutter und der Grossonkel (väterlicherseits) wurden vor Jahren wegen einer stark vergrösserten Milz und Ikterus splenektomiert. Nach der Milzexstirpation waren sie beschwerdefrei. Der Bruder des Vaters leidet seit Jahren an ähnlichen, aber weniger ausgeprägten Beschwerden. Beim Vater des Knaben trat im Alter von 34 Jahren der erste hämolytische Schub auf, die Mutter ist gesund

8jähriger körperlich und geistig altersgemäss entwickelter Knabe in gutem Allgemein- und Ernährungszustand Haut und Skleren subikterisch, Herztöne regelmässig mit einem systolischen Anämiegeräusch, Lunge perkutorisch und auskultatorisch ohne Besonderheiten. Abdomen weich, Appendektomienarbe im rechten Unterbauch. Leber nicht palpabel, Milz 2 QF vergrössert, palpabel Neurologisch unauffällig. Röntgenuntersuchung: Schädel. normale Verhältnisse. Thorax: Befund innerhalb der Norm, normale Luftverteilung. Normales perorales Cholezystogramm bei recht tief stehender Gallenblase. Laborbefunde: siehe Tabelle I

Eine Behandlung war zu dieser Zeit noch nicht notwendig, da auch ohne Bluttransfusionen die Hämoglobinwerte zwischen 11 7 und 12 g%/ lagen und der Knabe

Tabelle I Klinische Befunde des Patienten A. F vor und nach *Splenektomie*[1]

	Vor Splenektomie				Nach Splenektomie	
	2.8.1970	1.6.1972	7.2.1973	1.5 1973	4 9 1973	25.4.1975
Hb g%	11 7	11 7	12,0	7,0	17,0	16,6
Ery × 10⁶/µl	3,65	3,21	3,57	1,51	5,41	5,27
Leukozyten/µl	8 400	3 700	8 200	10 000	6 300	8 600
Retikulozyten, ‰	35	18	41	178	12	8
Haptoglobin	vermindert	vermindert	vermindert			
Bilirubin ges. mg%	3,5	4 0	2,5	1,5	0,7	
dir., mg%	0,4	1,2	1 44	0,8	0,5	
Coombs-Test (dir)	negativ	negativ	negativ			
Osmot. Resistenz, % NaCl		0 46–0,36	0,46–0,38			
Milz, QF palpabel	1	1	1	2 3		
Ery-Überlebenszeit (^{51}Cr)		14 Tage vermehrte Hämolyse über der Milz				

[1] Splenektomie am 25.6.1973

Phosphatanalyse Nach Erprobung einer Anzahl bewährter Bestimmungsmethoden für den Gesamtphosphorgehalt [8, 11 13, 22] wurde eine modifizierte Mikromethode erarbeitet, die im Bereich von 0,02 bis 25 µg Phosphor dem Beerschen Gesetz gehorcht. Durch Exposition in Joddampf wurden die einzelnen Phospholipidfraktionen lokalisiert, mit Bleistift markiert und nicht gebundenes Jod mit Warmluft entfernt. Dann wurden die Einzelfraktionen von der Platte geschabt, in 12-ml-Schliffröhrchen mit aufgesetzter Rückflusskappe mit 0,7 ml eines Gemisches aus 70%iger Perchlorsäure und 96%iger Schwefelsäure (1+1 Volumteile: Merck, suprapur) 2 h im Aluminiumheizblock bei 160–180 °C erhitzt, bis die vorübergehend farblos gewordene Flüssigkeit durch freigesetztes Chlor eine gelbgrüne Färbung annahm, abgekühlt und mit 4 ml einer frisch bereiteten 1%igen Lösung von Ammoniumheptamolybdat versetzt. Zu jeder dieser Fraktionen wurden 0,2 ml Fiske & Subbarow Reduktionsreagens (1-Amino-2-naphthol-4-sulfonsäure: Sigma, 1,0 g in 6,3 ml H_2O) zugesetzt und 10 min im kochenden Wasserbad erhitzt. Nach dem Abkühlen wurde der gebildete blaue Phosphomolybdatkomplex je nach Farbintensität mit 1,5 ml Isobutanol extrahiert und im Spektralphotometer bei 785 nm gegen Isobutanol gemessen. Phosphatspuren an den Glasgefäßen (Spülmittel, Tabakrauch) sind eine häufige Fehlerquelle dieser hochempfindlichen Mikromethode. Wir bevorzugen daher Chromschwefelsäure zur Reinigung der Arbeitsgefäße [21].

Radioaktivitätsbestimmung Zur Kontrolle und Lokalisation der radioaktiven Fraktionen wurde auf das fertig entwickelte Dünnschichtchromatogramm ein Röntgenfilm geklebt und 1–2 Wochen exponiert. Ausreichend genaue Meßwerte der Radioaktivitätsverteilung erhält man vom markierten Chromatogramm durch Auswertung mit dem Dünnschichtscanner (Berthold-Frieseke) vor der Analyse des Phosphorgehaltes der einzelnen Flecken. Exakte und gut reproduzierbare Ergebnisse werden erhalten, wenn man die radioaktiven Fraktionen von einer Parallelplatte in Packardgläschen schabt, dann mit Hilfe von Glaskugeln und einem Wirbelmixer fein zerreibt und in je 10 ml eines Gelszintillators im Flüssigkeitsszintillationszähler (z.B. Packard TRI-CARB) mißt. Folgende Szintillatormischung hat sich für unsere Versuche bewährt: 1000 ml Toluol, 0,15 g Dimethyl-POPOP, 5,50 g PPO, 40,00 g Cab-O-Sil (Eastman). Die gemessenen Aktivitäten wurden auf den Zeitpunkt des Versuchsbeginns extrapoliert und als 'spezifische Aktivitäten' auf den analytisch bestimmten Phosphorgehalt des Lipids (in µg) bezogen.

Membranenzyme Die Untersuchung der Ligase-Transferase-(AcTF) und Lysophospholipase-(LPL)-Aktivität erfolgte mit Hilfe radioaktiv markierter Substrate: 1-^{14}C-Ölsäure bzw. 1-Acyl-^{14}C-Lysolecithin in einem inhomogenen System bei Substratsättigung [6, 7]. Beide Enzyme sind im Gefriervollhämolysat partikelgebunden, Ölsäure ist wenig löslich und Lysolecithin bildet Micellen. Beide Substrate werden fast vollständig an die Membranen adsorbiert und besetzen dabei auch deren nicht enzymtragende Strukturen, wodurch der geringe relative Umsatz erklärt ist.

Ligase-Transferase (Acyl-CoA-Ligase = EC 6.2.1.3, Acyl-CoA-Lysolecithin-O-Acyltransferase = EC 2.3.1.?) 1-^{14}C-Ölsäure 35 nmol = 20 nCi, Lysolecithin 50 nmol, CoA 65 nmol, ATP 10 µmol, $MgCl_2$ 10 µmol. Enzym = 100 µl Hämolysat, entsprechend $5 \cdot 10^{8}$ Erythrozyten, 0,1 M Phosphatpuffer (pH 7,4) ad 1 ml Endvolumen. Inkubation 60 min bei 37 °C.

Lysophospholipase (Phospholipase B, Lysolecithinacylhydrolase = EC 3.1.1.5).

(Retikulozyten) und schwerste (Normozyten) Fraktion für die Untersuchungen verwendet.

Die Enzymaktivitäten der Glykolyse des Pentosephosphatzyklus und der GR wurden photometrisch nach einer Mikromethode aufgrund der Extinktionsänderungen bei $NADH_2$ bzw. $NADPH_2$ Konzentrationsänderungen gemessen [3]. Da die aus Hämolysaten bestimmten GR Aktivitäten häufig nur einen Bruchteil der insgesamt verfügbaren Gesamtaktivität darstellen wurde zu jeder Bestimmung auch die Aktivierung des Enzyms durch sein Koenzym Flavinadenindinukleotid (FAD) gemessen [1b, 2]

GSH und seine Stabilität gegen hämolytische Noxen wurde mit Hilfe von Acetylphenylhydrazin und des sulfhydrilspezifischen Farbstoffes 5,5 Dithiobis-(2-nitrobenzoesäure) bestimmt [1a, 4]

³²P Einbau in Phospholipide Pro Versuch wurde in drei Potter Elvehjem Homogenisatoren mit Teflonpistill (5 ml) je 1 ml Inkubationspuffer pipettiert und im Lyophilisator oder Vakuumtrockenschrank zur Trocknung gebracht. Dieser Inkubationspuffer bestand aus einer 5% Lösung von Humanalbumin, die im Milliliter 100 μmol NaCl 25 μmol NaH_2PO_4 H_2O 25 μmol $NaHCO_3$, 20 mol Glukose, 5 μmol $MgSO_4$ $7H_2O$ und 5 μmol KCl enthielt und mit NaOH auf pH 7,35 eingestellt war. Gegen die Verwendung des autologen Patientenplasmas für die Inkubation bei Phospholipideinbaustudien spricht der oft rasche aber variable Austausch von Membranphosphatiden gegen solche aus dem Plasma [19] In die vorbereiteten Reaktionsgefässe wurde 0,5–1 mCi $H_3{}^{32}PO_4$ (NEN trägerfrei in 1 ml H_2O) pipettiert, die Puffersalze wurden darin gelöst und zuletzt wurden 0,3 ml Erythrozytensediment (5 10⁹ Zellen) zugefügt. Anschliessend wurde 1 h bei 37 °C im Schüttelwasserbad inkubiert und die Reaktion dann im Eisbad gestoppt

Phospholipidextraktion Nach Inkubationsende wurden die Homogenisatoren bei 1000–1500 g 15 min zentrifugiert der radioaktive Überstand wurde mit einer Pasteurpipette vorsichtig abgesaugt und verworfen Anschliessend wurden die Erythrozytensedimente dreimal unter denselben Bedingungen mit eiskalter Inkubationslösung ohne Albuminzusatz gewaschen Um Verklumpung bei der Extraktion zu vermeiden, wurden die Rückstände lyophilisiert und mehrmals mit dem insgesamt 25–50fachen Volumen eines Gemisches aus 11 Teilen Isopropanol [ ] und 7 Teilen Chloroform nach ROSE und OKLANDER [20] extrahiert. Die Rückstände wurden jeweils bei 3000 g 15 min sedimentiert, die Extrakte mit Hilfe von Pasteurpipetten in je einem 25-ml Birnenkölbchen vereinigt und mit einem Vakuumrotationsverdampfer bei 40 °C unter Stickstoff bis fast zur Trocknung eingedampft

Phospholipidchromatographie Der Extraktionsrückstand in jedem Kölbchen wurde in 1–2 Tropfen Chloroform/Methanol 2+1 Volumteile) gelöst und mit einer Kapillare auf den Startpunkt einer DC Kieselgel Fertigplatte (Merck) aufgetragen. Um die quantitative Überführung des Verdampfungsrückstandes auf die Platte zu gewährleisten, wurde das Kölbchen auf die gleiche Art weitere fünfmal ausgespült. Anschliessend wurde die Platte in der ersten Dimension 6 h in einem alkalischen Fliessmittel, bestehend aus 1,2 Dichloräthan/Methanol Isobutanol 30% Ammoniak (45+30+15+10 Volumteile) entwickelt, über Nacht im Vakuumexsikkator getrocknet, dann 6 h in der zweiten Dimension in einem sauren Gemisch aus 1,2 Dichloräthan/Methanol/Isobutanol/Eisessig/Wasser (45+30+15+5+5 Volumteile) entwickelt [15]

Tabelle II. Resultate der Erythrozytenenzym- und Substratbestimmungen

	Patient	Normalwerte (n = 25)
Embden-Meyerhof-Weg		
HK, U/g Hb	0,54	0,64 ± 0,27
F-6-PK, U/g Hb	12,07	12,60 ± 1,66
PK, U/g Hb	5,97	5,38 ± 1,67
ATP µmol/g Hb	5,34	4,05 ± 0,38
ADP µmol/g Hb	0,64	0,59 ± 0,08
AMP µmol/g Hb	0,15	0,08 ± 0,01
Laktatbildung/h µmol/g Hb	976,70	932,00 ± 211,00
Pyruvatbildung/h µmol/g Hb	66,01	53,30 ± 1,50
Pentose-Phosphat-Zyklus		
G-6-PDH (WHO), U/g Hb	15,85	13,55 ± 1,59
6-PGDH, U/g Hb	*9,08*	*8,60 ± 1,08*
GR, U/g Hb	3,19	5,68 ± 1,52
GR + FAD U/g Hb	3,62	7,44 ± 1,63
GSH, µmol/g Hb	10,13	6,57 ± 1,04
GSH-Stabilität, % Abfall	46,30	20,70 ± 5,30
Membranenzyme		
AcTF nmol/10^{10} Ery/min	27,00	107,00 ± 26,00
LPL, nmol/10^{10} Ery/min	131,00	122,00 ± 38,00

Tabelle III. Familienbefunde der GR und der Stabilität des GSH

	Normal (n = 25)	Patient	Vater	Onkel	Gross-mutter	Gross-onkel
GR, U/g Hb	5,68 ± 1,52	3,19	1,98	3,56	4,14	3,86
Verminderung gegen «Normal», %		–43,8	–65,2	37,2	27,1	32,0
GR + FAD U/g Hb	7,44 ± 1,63	3,62	2,90	3,82	4,21	3,89
Verminderung gegen «Normal» %		51,3	–61,1	–48,7	–43,4	–47,8
GSH-Stabilität, % Abfall	20,7 ± 5,3	46,3	37,6	37,2	34,6	39,2
Splenektomie	nein	ja	ja	nein	ja	ja

1 Acyl ^{14}C Lysolecithin 50 nmol = 5 nCi, $MgCl_2$ 10 µmol, Enzym = 100 µl Hämolysat, entsprechend 5·10⁸ Erythrozyten 0,1 M Phosphatpuffer (pH 7,4) ad 1 ml Endvolumen. Inkubation 60 min bei 37 °C.

Zur Aktivitätsbestimmung beider Enzyme wurde die Inkubationslösung anschliessend mit Isopropanol und Chloroform 7+3 ml [20] unter Trägerzusatz (100 µl Phospholipidgemisch entsprechend dem Extrakt aus 10⁹ Erythrozyten, 450 nmol Lysolecithin, 70 nmol Ölsäure) extrahiert und im Wasserstrahlvakuum bei 30 °C eingedampft. Der Rückstand wurde quantitativ mit Chloroform/Methanol 2+1 auf eine Kieselgel DC Fertigplatte (Merck) strichförmig aufgetragen und in Chloroform/Methanol/Wasser 65+40+8 (v/v) bis zum oberen Rand der Platte entwickelt. Nach dem Anfärben der einzelnen Fraktionen in Joddampf wurden die Banden von Lysolecithin und Ölsäure markiert, von der Patte geschabt, in Packard-Messgläschen übergeführt mit Hilfe von Glaskugeln darin fein zerrieben und in einem Cab-O Sil-Gelszintillator im Flüssigkeitszintillationszähler gemessen. Bei der Auswertung wurde die umgesetzte Radioaktivität zur Substratkonzentration und Erythrozytenzahl im Hämolysat in Beziehung gesetzt [16, 18].

Ausbeuten und Reproduzierbarkeit Da jede Interpretation unserer Ergebnisse letztlich von der Ausbeute der chemischen Operationen und ihrer exakten Reproduzierbarkeit abhängt, wurden bei ausreichender Blutmenge jeweils 2 oder 3 Parallelansätze derselben Erythrozytenfraktion analysiert. Die Resultate der chemischen Bestimmungen allein und der Kombination mit Radioaktivitätsmessungen waren innerhalb einer Streubreite von maximal 10–15% reproduzierbar. Die mittlere Ausbeute der Phosphatanalysen von Phospholipid Standardgemischen lag bei 96,7±3,9%.

Ergebnisse

Unter den für den Erythrozytenstoffwechsel regulatorisch wirksamen Enzymen des glykolytischen Abbauweges wurden Hexokinase (HK) Phosphofruktokinase (F-6-PK) und Pyruvatkinase (PK) untersucht. Bei unserem Patienten A. F. und allen untersuchten Familienmitgliedern liegen die Werte dieser Enzyme im Normalbereich (Tab II). Damit im Einklang stehen die relativ hohen Werte der energieliefernden Adenosinphosphate und von Laktat und Pyruvat.

Glucose-6-Phosphat Dehydrogenase (G-6-PDH) und 6-Phosphogluconat Dehydrogenase (6-PGDH) als geschwindigkeitsbeschränkende Enzyme des Pentosephosphatzyklus zeigten bei keinem der untersuchten Familienmitglieder eine Aktivitätsverminderung, dagegen war die GR beim Patienten A. F. und vier nahen Blutsverwandten stark erniedrigt. Noch geringer war der mit dem spezifischen Koenzym FAD aktivierbare Anteil der GR bei allen fünf Familienangehörigen (Tab III). Auffallend ist der Anstau des GSH beim Patienten A. F. [vgl. 9] und die stark verminderte Glutathionstabilität, d. h. die Unfähigkeit des Erythrozyten sei-

dem eine verminderte GR-Aktivität, insgesamt geringe Aktivierbarkeit durch das Koenzym FAD und verringerte Stabilität des GSH gegen das hämolytisch wirkende Acetylphenylhydrazin festzustellen. Schon bei partieller Verminderung der GR-Aktivität wurde eine Vielzahl hämatologischer (hereditäre hämolytische Anämie, Panzytopenie), neurologischer und anderer Syndrome (Oligophrenie) neben einer verstärkten Primaquin-Sensibilität beschrieben [23, 24]. Die Interpretation dieses Enzymmangels wird durch die klinische Heterogenität seiner Manifestationen und den wechselnden Einfluss der Riboflavinversorgung auf die Verfügbarkeit des GR-Koenzyms FAD erheblich erschwert.

Die von uns zusätzlich gefundenen Veränderungen an der Membran weisen auf eine komplexe Störung hin. Ihre teilweise Behebung durch die Splenektomie bekräftigt die Ähnlichkeit mit dem Defekt, der bei Kugelzellenanämie [16–18] für die Spontanhämolyse verantwortlich ist, da nach den bisher vorliegenden Erfahrungen der Symptomkomplex „GR-Mangel“ durch Splenektomie nicht beeinflusst werden kann. Bei dem von uns untersuchten Patienten liegen vermutlich zwei verschiedene, genetisch bedingte Defekte vor: nämlich die reduzierte Aktivität der GR und eine davon unabhängige Membranschädigung. Der Erbgang dieser kombinierten Störung ist bei der von uns untersuchten Familie autosomal-dominant.

Dank

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Zusammenfassung

Glutathionreduktaseaktivität und Parameter des Phospholipidstoffwechsels von Erythrozytenmembranen wurden bei einer Familie mit hereditärer hämolytischer Anämie bestimmt. Dabei wurde eine deutlich verminderte Aktivität der Glutathionreduktase und verringerte Stabilität des reduzierten Glutathions zusammen mit gesteigertem Phosphatumsatz der Phospholipide und herabgesetzter Aktivität des membranstabilisierenden Enzyms Lysolecithin-Acyltransferase festgestellt. Eine Splenektomie führte in allen Fällen zu einer Normalisierung. Die Untersuchungen dieser komplexen funktionellen Störung ergaben Anhaltspunkte für einen autosomal-dominanten Erbgang.

Tabelle IV Phospholipidgehalt und -phosphatumsatz in Erythrozyten des Patienten A.F vor und nach Splenektomie

	Phospholipidverteilung, µmol/10[11] Erythrozyten		Phospholipidumsatz, dpm/µg Lipid P		
	Patient	Normalwerte (n = 20)	Patient vor Splen-ektomie	Patient nach Splen-ektomie	Normalwerte (n = 20)
2,3-DPG und Phosphopeptide	0,36	0,57 ± 0 17	128 40[illegible]	68 206	25 000 ± 9 000
SphC	8,18	7 64 ± 1,33	5	3	3 ±
PC	7 42	8,31 ± 1,85	1	12	18 ± 1
PI	0,22	0,17 ± 0,05	3 058	4 189	6 500 ± 4 000
PS	3,82	3,30 ± 0,57	8[illegible]1	[illegible]97	85 ± 35
PE	8,96	7,36 ± 1 08	15	18	[illegible]5 ± 15
PA	0,54	0,58 ± 0,16	44 987	4 418	12 000 ± 5 500

2,3-DPG = 2,3-Diphosphoglycerat SphC = Sphingomyelin PC = Lecithin PI = Phosphatidylinosit PS = Phosphatidylserin PE = Phosphatidyläthanolamin PA = Phosphatidsäure.

nen GSH Spiegel unter Einwirkung einer 2 Acetyl Phenylhydrazin Bela stung aufrechtzuerhalten.

Die Phosphatumsatzaktivität der Membranphospholipide gemessen am Einbau anorganischen [32]P-Orthophosphats über das reichlich vor handene ATP diente uns als Mass für die Fragilität und Strukturerneuerung der Membran Besonders auffällig war in unserem Fall die um eine Grössenordnung gesteigerte Umsatzaktivität des PS, die nach der Sple nektomie um 64% zurückging, während die ebenfalls hohe PA Aktivität annähernd gleich blieb (Tab IV)

Von den membranstabilisierenden lysolecithinabbauenden Enzymsy stemen war die Aktivität der LPC Acyltransferase (AcTF) um 75% ge genüber der Norm vermindert, während der Wert der LPL unauffällig war (Tab II)

Diskussion

Neben den biochemischen Anzeichen einer Membranschädigung der Erythrozyten sind beim Patienten A F und mehreren Familienmitglie

dem eine verminderte GR Aktivität, insgesamt geringe Aktivierbarkeit durch das Koenzym FAD und verringerte Stabilität des GSH gegen das lytisch wirkende Acetylphenylhydrazin festzustellen. Schon bei partieller Verminderung der GR-Aktivität wurde eine Vielzahl hämatologischer (hereditäre hämolytische Anämie, Panzytopenie), neurologischer und anderer Syndrome (Oligophrenie) neben einer verstärkten Primaquin-Sensibilität beschrieben [23, 24]. Die Interpretation dieses Enzymmangels wird durch die klinische Heterogenität seiner Manifestationen und den wechselnden Einfluss der Riboflavinversorgung auf die Verfügbarkeit des GR Koenzyms FAD erheblich erschwert.

Die von uns zusätzlich gefundenen Veränderungen an der Membran weisen auf eine komplexe Störung hin. Ihre teilweise Behebung durch die Splenektomie bekräftigt die Ähnlichkeit mit dem Defekt, der bei Kugelzellenanämie [16–18] für die Spontanhämolyse verantwortlich ist, da nach den bisher vorliegenden Erfahrungen der Symptomkomplex «GR-Mangel» durch Splenektomie nicht beeinflusst werden kann. Bei dem von uns untersuchten Patienten liegen vermutlich zwei verschiedene, genetisch bedingte Defekte vor: nämlich die reduzierte Aktivität der GR und eine davon unabhängige Membranschädigung. Der Erbgang dieser kombinierten Störung ist bei der von uns untersuchten Familie autosomal-dominant.

Dank

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Zusammenfassung

Glutathionreduktaseaktivität und Parameter des Phospholipidstoffwechsels von Erythrozytenmembranen wurden bei einer Familie mit hereditärer hämolytischer Anämie bestimmt. Dabei wurde eine deutlich verminderte Aktivität der Glutathionreduktase und verringerte Stabilität des reduzierten Glutathions zusammen mit gesteigertem Phosphatumsatz der Phospholipide und herabgesetzter Aktivität des membranstabilisierenden Enzyms Lysolecithin-Acyltransferase festgestellt. Eine Splenektomie führte in allen Fällen zu einer Normalisierung. Die Untersuchungen dieser komplexen funktionellen Störung ergaben Anhaltspunkte für einen autosomal-dominanten Erbgang.

Tabelle IV Phospholipidgehalt und phosphatumsatz in Erythrozyten des Patienten A.F vor und nach Splenektomie

	Phospholipidverteilung, µmol/10^{11} Erythrozyten		Phospholipidumsatz, dpm/µg Lipid-P		
	Patient	Normalwerte (n = 20)	Patient vor Splen-ektomie	Patient nach Splen-ektomie	Normalwerte (n = 20)
2,3-DPG und Phosphopeptide	0,36	0,57 ± 0,17	128 402	68 206	25 000 ± 9 000
SphC	8,18	7 64 ± 1,33	5	3	3 ± 2
PC	7 42	8,31 ± 1,85	21	12	18 ± 12
PI	0,[illegible]	0 17 ± 0,05	3 058	4 189	6 500 ± 4 000
PS	3,82	3,30 ± 0,57	821	297	85 ± 35
PE	8,96	7,36 ± 1 08	15	18	25 ± 15
PA	0,54	0,58 ± 0,16	44 987	4 418	12 000 ± 5 500

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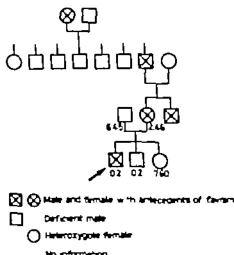


Fig. 1. Family tree of the propositus (↗). The erythrocyte G-6PD activities of the propositus brother sister and parents, are indicated (normal values 6.1 ± 0.7)

tion and kinetic studies of the erythrocyte enzyme [ ], isoelectrofocusing in acrylamide ampholine gel [4], immunological titrations by electroimmunodiffusion with specific staining of the immunoprecipitate peaks for G-6PD activity [4, 5]. The assays of G-6PD activity were performed by subtracting 6-PGD activity from the activity of G-6PD and 6-phosphogluconate dehydrogenase assayed together [16]. The results were expressed in IU at 30 °C/mg of proteins for leukocytes and platelets and per gram of hemoglobin for ed blood cells.

Clinical observation and family study (fig. 1). The propositus, 6-year-old boy was admitted to the hospital because of an episode of acute hemolysis of mild intensity after ingestion of fava beans. A similar episode was noted 2 years ago. His brother and his sister were clinically normal, but favism features were found in the mother the maternal uncle, the maternal grandfather and great grandmother 2 months after the acute episode the propositus had no clinical or biological features of chronic hemolysis.

Results

G-6PD activity was reduced to less than 4 of normal in erythrocytes of the brothers, 13%/ in their platelets and 27% in their leukocytes (table I fig. 1). The mother had a partial enzyme deficiency in erythrocytes (2.46 IU/g Hb i.e. 40% of normal) Starch gel electrophoresis (fig. 2)

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Favism in a Portuguese Family Due to a Deficient Glucose-6-Phosphate Dehydrogenase Variant of 'Canton' or 'Canton-Like' Type[1]

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Key Words Canton-type of G-6PD Erythrocyte enzymes Favism G-6PD variants

Abstract In a Portuguese boy with favism G-6PD deficiency was found. Deficiency was due to a G-6PD variant close or identical to Canton-type G-6PD The muted protein had a lowered catalytic activity and, furthermore, was unstable. The post-translational modifications undergone by this deficient G-6PD variant are described.

Hemolytic crises following ingestion of fava beans may occur in subjects with various types of glucose-6-phosphate dehydrogenase (G-6PD) deficiency especially with the Mediterranean [8, 13 14] and Canton [1 10] types.

We describe herein a Portuguese family with favism due to G-6PD deficiency The enzyme deficiency was associated with the presence in the deficient erythrocytes of a G-6PD variant close or identical to the variant Gd (–) Canton

Material and Methods

The substrates of the enzymatic reactions were furnished by Boehringer Mannheim and Sigma C. C. Ion exchangers came from Pharmacia. The enzymatic reactions were measured with a Zeiss PMQII spectrophotometer and an Aminco photofluorimeter both connected to recorders. Normal and deficient G-6PD have been studied by methods previously described blood cell separation [4] partial purifica

[1] This work was supported by a grant of Inserm (ATP 8 74 29)

Table I. Kinetic characteristics of the G-6PD variant studied and of Gd ( ) Canton variant

	Propositus	Normal values, mean ± 1 SD	Gd (–) Canton[1]
Enzyme activity			
Red blood cells, IU/g Hb	≃0.2	6.1 ± 0.7	7.3 ± 1.9
Platelets, IU/mg protein	0.025, 13%	0.19 ± 0.045	
Leukocytes, IU/mg protein	0.184, 27%	0.68 ± 0.19	35.3 ± 187
Electrophoretic mobility % of normal (starch gel–Tris-Cl pH 8)	107	100	106–108
Michaelis constant, μM			
G-6P	32	45.9 ± 5.8	22.6 ± 5.3
NADP+[2]	14	11 ± 2	
Ki NADPH, μM[3]	43.5	25 ± 3	
Inhibition by ATP %	34	30–50	
Utilization of the substrate analogues, %			
2 d-G6P	18.5	<6	11.4 ± 6.5
Gal-6P	16.5	<6	11.2 ± [illegible]8
Deamino-NADP+	140	40–60	143.5 ± 4.5
pH curve, optimal pH	slightly biphasic 7 9.2	truncate 8.5	biphasic 6.5–7 and 9.5
Thermal inactivation reaction velocity constant at 48 °C (10^3 s[illegible])	unstable 71	<20	unstable
Activation energy of the enzymatic reaction, kcal/mol	19	10.4 ± 0.96	

The characteristics of Gd ( ) Canton variant are given by P[illegible]NICH and SUNGNATE [11].
In 50 mM Tris-Cl-buffer pH 7.3 containing 100 mM KCl, 4 mM $MgCl_2$ at 37 °C [15].
Measurements were made by fluorometry
Using the same buffers as that above, but by spectrophotometry at 30 °C.

showed that the deficient leukocytes and platelets from the propositus contained a fast variant (mobility at 107% of normal). G-6PD activity was not electrophoretically detectable in the deficient hemolysate. By electrofocusing the G-6PD active forms of the propositus's cells were also slightly more anodic than those of a control (fig. 3). Besides, the deficient leukocytes contained several enzymatic forms, especially anodic forms, while normal leukocytes always contain only a predominant band a [4]

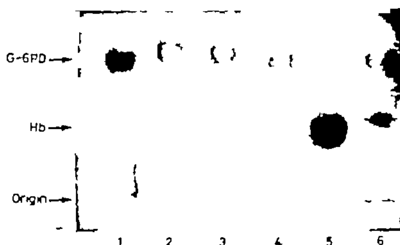


Fig 2 Starch gel electrophoresis in TEB pH 8 1 = Control granulocytes 2 = propositus's granulocytes 3 = propositus's platelets 4 = control platelets 5 = propositus's hemolysate 6 = control hemolysate.

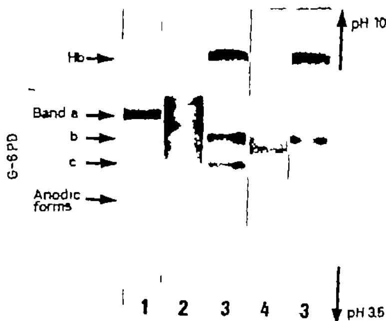


Fig 3 Electrofocusing pattern in acrylamide ampholine gel of normal and deficient G-6PD from leukocyte and red blood cells. 1 = Control leukocytes 2 = propositus's leukocytes 3 = control hemolysate 4 = partially purified erythrocyte enzyme from the propositus.

the total red cell population from a hemizygous male [12] Table I compares the properties of the propositus s G-6PD to those of G-6PD Canton [10, 11] the level of the enzyme deficiency in the blood cells as well as the electrophoretic and kinetic characteristics of both enzymes were identical. Furthermore, favism has been also reported in West China [1] Of course, the structural analysis of muted proteins only could assure of the identity of Canton-type G-6PD with the variant studied herein. Nevertheless, the colonial story of Portugal might account for an Asiatic ancestress in a Portuguese family Immunological studies showed that muted G-6PD had a lowered molecular specific activity that accounts mainly for the enzyme deficiency in leukocytes. Furthermore, this variant was unstable and this unstability explained the aggravation of the deficiency in platelets and red blood cells.

The slight decrease of the molecular specific activity as well as the modifications of the electrofocusing pattern between leukocytes and red blood cells were an example of the post-translational modifications that G-6PD undergoes after its synthesis [4] An unstable G-6PD variant could also age more quickly than the normal enzyme and, therefore, in a patient with unstable G-6PD variant, aged enzymatic forms might be found in younger cells (such as the leukocytes) than in a control. This probably explains the presence of anodic forms of muted G-6PD in the propositus s leukocytes. In the propositus s red blood cells, however only one predominant form was found corresponding to band b This finding was not due to the partial purification, that has been previously shown to not change the electrofocusing pattern [4] The higher unstability of the anodic forms, disappearing first from deficient red blood cells, might account for such a result. We have previously reported the same phenomenon of accelerated molecular aging of several other unstable G-6PD variants [6, 7]

In conclusion, this work, beside the surprising detection of Canton-type G-6PD in a Portuguese family describes the molecular mechanism of the enzyme deficiency associated with Canton-type G-6PD and emphasizes the peculiar pattern of the molecular aging of an unstable muted enzyme.

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Table II Immunological titration of G-6PD in various blood cells of the propositus

	Enzymatic activity % of normal	Immunological reactivity % of normal	Enzymatic activity / Immunological reactivity % of normal leukocyte ratio[1]
Leukocytes	_7	69	39 (100)
Platelets	13	38	34.5 (86 ± 6)
Red blood cells	∼3	8.7	∼34.5 (62 ± 6)

[1] The normal values, in parentheses, are given ± 1 SD

In contrast, the band c and the anodic forms found in normal hemolysates as well as in normal partially purified preparations from erythrocytes was lacking in the propositus's preparation.

The main kinetic characteristics of the deficient G-6PD (table I) were a slightly decreased Michaelis constant for glucose-6-phosphate (32 μM), an increased inhibition constant by NADPH with respect to $NADP^+$ (43.5 μM), an abnormal utilization of the substrate analogues (the glucose-6-phosphate analogues as well as the NADP analogues), an abnormal pH curve, slightly biphasic, with two optimal pH of 7 and 9 2 a thermo-unstability and an increased activation energy of the enzymatic reaction (19 kcal/mol)

The immunological titrations, compared with the enzymatic titrations, showed that the molecular specific activity (represented by the ratio of enzymatic activity to immunological reactivity) was reduced to 39% of normal in leukocytes, 34% of normal in platelets and red blood cells from the propositus (table II) Consequently the concentration of G-6PD-antigen was slightly decreased in the leukocytes, markedly decreased in the platelets and almost null in the red blood cells.

Discussion

In the family presented herein two women have had episodes of mild hemolysis after ingestion of fava beans (fig 1) The random inactivation of the X chromosome in the heterozygous females [9] account for a mosaicism with two cell populations, one normal and the other deficient. The G-6PD-deficient red cell population is sensitive to hemolysis as well as

the total red cell population from a hemizygous male [12]. Table I compares the properties of the propositus's G-6PD to those of G-6PD Canton [10, 11]: the level of the enzyme deficiency in the blood cells as well as the electrophoretic and kinetic characteristics of both enzymes were identical. Furthermore, favism has been also reported in West China [1]. Of course, the structural analysis of muted proteins only could assure of the identity of Canton-type G-6PD with the variant studied herein. Nevertheless, the colonial story of Portugal might account for an Asiatic ancestress in a Portuguese family. Immunological studies showed that muted G-6PD had a lowered molecular specific activity that accounts mainly for the enzyme deficiency in leukocytes. Furthermore, this variant was unstable and this unstability explained the aggravation of the deficiency in platelets and red blood cells.

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Granulocyte Function in Untreated Acute and Chronic Granulocytic Leukemia

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Key Words. Infection in leukemia · Leukemia · Myeloperoxidase · Neutrophil function · Nitro-blue tetrazolium test

Abstract. Untreated patients with acute granulocytic leukemia showed impairment of microbicidal activity and, in one, this was associated with myeloperoxidase deficiency and staphylococcal infection. In chronic granulocytic leukemia, there was no significant impairment of microbial killing. However, reduction in the capacity to reduce nitro-blue tetrazolium indicated some disturbance of neutrophil function in this disorder.

Patients with acute leukemia are liable to infection both at presentation and during treatment. This is usually ascribed to neutropenia [3–5, 32], although some attention has been paid to abnormalities of granulocyte function [8, 10–15, 26, 27]. In chronic granulocytic leukemia, there is little clinical evidence of increased susceptibility to infection; nonetheless, abnormalities of granulocyte function occur in the course of this disease [11, 25, 29, 35, 36].

In many of these cases, granulocyte function has been assessed in patients treated with antileukemic agents. As it is not certain whether those agents may be responsible for some of the inconsistencies reported between various groups of patients, we have investigated granulocyte function in untreated acute and chronic granulocytic leukemia.

Materials and Methods

Patients. There were seven patients with acute granulocytic leukemia and ten with chronic granulocytic leukemia (table I, II). Cases with acute leukemia in whom

Table I Patients with acute granulocytic leukemia

Case No.	Sex/age		WBC $\times 10^3/\mu l$	Neutrophils, %	Blast cells %	Evidence of infection	Bacterial killing[1]	Fungal killing[1]	MPO score	NBT reduction %
1	M	17	.5	28	8	none	5.2	34	364	60
	M	36	6.9	57	5	none	12.3	18	ND	66
3	M	4	18 6	30	32	none	9.3	22	290	50
4	F	43	7 7	48	7	none	5.9	ND	ND	28
5	F	12	4 4	10	83	URTI	4	2	356	65
6	M	70	4 0	23	27	none	44	8	376	40
7	F	73	3.1	39	43	Septic lesion at sternal marrow biopsy site	54	7	182	3.

In each patient the bone marrow showed heavy infiltration by primitive granulocyte precursors.
MPO = Myeloperoxidase NBT = nitro-blue tetrazolium ND = not done URTI = upper respiratory tract infection.

[1] Bacterial and fungal killing using patient s cells suspended in normal serum. Bacterial killing expressed as percent *survival* of *S. aureus*, fungal killing as percent *killing* of *Candida albicans*.

monocytes exceeded 10% of the number of mature neutrophils were excluded from the study In no instance had the patient received any treatment prior to investigation of granulocyte function. One patient with acute granulocytic leukemia (case No. 7) had myeloperoxidase deficiency of her neutrophils, with 38% of the cells showing no myeloperoxidase activity She developed infection with *Staphylococcus aureus* at the site of sternal marrow puncture and was treated with cloxacillin. She subsequently developed fatal fulminant enterocolitis. Another patient (case No. 5), had a transient simple upper respiratory infection, presumably viral in origin. Otherwise, no clinical evidence of active infection was found in any patient at the time of study

Methods. Routine blood investigations were performed according to standard methods [9] or on a Coulter Counter Model S. Leukocyte alkaline phosphatase was determined according to HAYHOE *et al.* [13].

Leukocytes for microbicidal experiments were prepared from heparinized blood by sedimentation of red cells with dextran and removal of white cell-rich plasma. The white cells were then centrifuged (450 *g*) and washed gently with Hanks buffered salt solution (HBSS) they were resuspended in HBSS and the total white cells were counted. The total neutrophil count was calculated from the differential count and the strength of the white cell suspension adjusted to give a concentration of 10^7 mature neutrophils/ml. The final concentration of mature neutrophils after dilution in HBSS and serum, and addition of microorganisms was 5×10^6/ml. Contamination

Table II. Patients with chronic granulocytic leukemia

Case No.	Sex/age	WBC $10^3/\mu l$	Ph[1]	LAP	Bacterial killing[1]	Fungal killing	NBT[1] reduction %
8	M 51	232	+	ND[1]	1.6	9	8
9	F 40	264	+	<10	ND	22	33
10	M 53	296	+	<10	1.5	32	14
11	M 83	13.7	–	ND	1.4	39	25
12	F 68	18.9	ND	ND	0.2	15	66
13	M 63	6.4	ND	<10	0.3	11	39
14	M 91	46	–	13	0.1	13	20
15	F 81	192	+	<10	0.7	7	9
16	M 23	135	+	<10	1.6	12	37
17	M 52	37	+	ND	1.8	4	17

In all cases, the bone marrow showed marked granulocyte hyperplasia with well differentiated granulopoiesis. LAP = Leucocyte alkaline phosphatase score.

[1] See legend to table I.

by the small numbers of monocytes present and by primitive myeloid cells is unlikely to have influenced results as monocytes are relatively inefficient in microbial killing [2] and primitive granulocytes have little or no phagocytic capacity [1, 34]. White cells were obtained similarly from an ABO-compatible normal healthy control donor for each patient studied. Serum was also obtained from each patient and each control subject.

Bactericidal capacity of neutrophils was tested against *S. aureus*, strain 502A, after 90 and 180 min of incubation of cells with bacteria [28]; results at 180 min only have been used for analysis. Fungicidal activity was tested against *Candida albicans* [20].

The nitro-blue tetrazolium test was carried out as described by PARK *et al.* [23] and PARK and GOOD [24]. Myeloperoxidase was assessed using a cytochemical technique [16]; a simple scoring system was followed to obtain a quantitative measure of enzyme activity (100 consecutive neutrophils were 'scored' 0–4 on the basis of intensity of staining and the scores summed) giving a maximum range of 0–400 for the myeloperoxidase score.

Results

Acute granulocytic leukemia. Bacterial killing was significantly reduced in acute granulocytic leukemia (table III); the significant difference was due to three patients (cases No. 5, 6, 7) showing marked impairment of bactericidal capacity (table I); the remaining four patients had a nor

Table I Patients with acute granulocytic leukemia

Case No.	Sex/age	WBC $\times 10^3/\mu l$	Neutrophils, %	Blast cells %	Evidence of infection	Bacterial killing[1]	Fungal killing[1]	MPO 'score'	NBT reduct. %
1	M 17	.5	28	8	none	5.2	34	364	60
2	M 36	6.9	57	5	none	12.3	18	ND	66
3	M 42	18 6	30	32	none	9.3	2	290	58
4	F 43	7 7	48	7	none	5 9	ND	ND	21
5	F 12	4 4	10	83	URTI	42	2	356	65
6	M 70	4 0	3	27	none	44	8	376	40
7	F 73	3 1	39	43	Septic lesion at sternal marrow biopsy site	54	7	182	32

In each patient the bone marrow showed heavy infiltration by primitive granulocyte precursors
MPO = Myeloperoxidase NBT = nitro-blue tetrazolium ND = not done URTI = upper respiratory tract infection.

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tion in candidacidal capacity compared with normal (table II III) and these three patients also had rather low potential for NBT reduction on stimulation with endotoxin (table II). There was no evidence of an effect of patient's serum on microbial killing (table III) and myeloperoxidase activity was normal in all ten patients.

Compared with 19 normal controls (55 ± 16.7%) there was a significant ($p<0.001$) decrease in the capacity of chronic granulocytic leukemic neutrophils to reduce NBT (25 ± 17.5%, 10 observations) and this decrease was more obvious in those individuals with the Ph chromosome (16 ± 9%, 6 observations). The numbers of cases are, however too small to allow statistical comparison of Ph^1-positive and Ph^1-negative patients.

Discussion

Three patients with acute granulocytic leukemia showed impaired fungicidal and bactericidal activity In one, myeloperoxidase deficiency probably played a part in decreasing microbicidal activity Myeloperoxidase is an important participant in the hydrogen peroxide-myeloperoxidase-halide system proposed by KLEBANOFF [17 18] and STOSSEL [33] and has a major role in the destruction of microorganisms. Congenital deficiency of this enzyme leads to undue susceptibility to infection [21] and defective microbicidal activity [22] Acquired myeloperoxidase deficiency is seen in some patients with acute granulocytic leukemia [8, 13] and in one instance this has been associated wiht abnormal microbicidal activity and liability to infection [10] It is notable that the only patient in the present series to develop clinically significant bacterial infection prior to any therapy also had myeloperoxidase deficiency

However myeloperoxidase deficiency is clearly not the only abnormality giving rise to impaired *in vitro* microbial killing since two other patients (cases No 5 6) exhibit similar impairment without myeloperoxidase deficiency The metabolic basis of the functional abnormality in these individuals is unknown. Inability to supply reducing potential through the NADP NADPH system is unlikely since the stimulated NBT reduction was within normal limits in these two patients. Impaired phagocytosis could be responsible and has not been excluded from this study However the evidence that phagocytosis in acute granulocytic leukemia is impaired, is conflicting, with some investigators showing defective phagocytosis [14 26] and others failing to demonstrate such a defect [7

Table III Microbial killing in acute and chronic granulocytic leukaemia

	Normal serum			Normal cells		
	patient cells	normal cells	p	patient serum	normal serum	p
Acute granulocytic leukemia						
Bactericidal capacity	24 6 ± 1 (7)	3 3 ± 3 0 (9)	<0.05	3.2 ± 1 9 (5)	3.0 ± 1 9 (6)	>0 4
Fungicidal capacity	15 ± 11 8 (6)	[illegible]2 ± 6.7 (8)	>0.[illegible]	18 ± 11.2 (14)	[illegible]1 ± 5.5 (4)	>0.3
Chronic granulocytic leukemia						
Bactericidal	1 1 ± 0.6 (9)	0.7 ± 0.6 (9)	>0.1	0.9 2.4 2.1 (3)	0.8, 0.9 1.9 (3)	
Fungicidal	16 ± 11.3 (10)	19 ± 4 7 (10)	>0.[illegible]	16 ± 5.6 (4)	19 ± 3 6 (4)	>0.2

Results for bacterial killing are presented as percent *survival* after 180 min exposure to neutrophils and results for fungal killing as percent organisms *killed*. Mean ± 1 SD of the mean. Numbers of observations are given in parenthesis.

mal capacity for destruction of *S aureus* One of the three patients (case No 7) showing clinical evidence of bacterial infection and impairment of bacterial killing also showed myeloperoxidase deficiency (table I) The capacity to kill *Candida albicans* was reduced in the same three patients who had impaired bactericidal capacity (table I) However taking the group as a whole there was no statistically significant difference between normal and acute granulocytic neutrophils (table III) In this series there was no difference between acute granulocytic leukemia serum and normal serum in respect of the support of microbicidal activity by normal cells (table III) Myeloperoxidase activity in 4 of 5 patients tested was normal including two patients showing impaired bacterial killing. The fifth patient (case No 7), who showed myeloperoxidase deficiency also showed evidence of impaired bacterial and fungal killing (table I) Stimulated nitro-blue tetrazolium reduction by mature neutrophils was not statistically significantly different from normal The mean value for seven patients was 49% (SD 15 7%) and for 19 normal controls 55 ± 16 7%.

Chronic granulocytic leukemia There was no evidence of impaired ability to kill *S aureus* (table III) There was no statistically significant difference in the ability of chronic granulocytic neutrophils to kill *Candida albicans* however three patients (case No 8, 15 17) showed reduc

tion in candidacidal capacity compared with normal (table II III) and these three patients also had rather low potential for NBT reduction on stimulation with endotoxin (table II). There was no evidence of an effect of patient's serum on microbial killing (table III) and myeloperoxidase activity was normal in all ten patients.

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30 31] Unlike GOLDMAN and TH'NG [12] we have not demonstrated an effect of serum from patients with acute granulocytic leukemia in inhibiting microbial killing. No obvious explanation is apparent for this discrepancy but an influence of previous therapy in their patients cannot be discounted.

Bacterial killing was normal in all patients with chronic granulocytic leukemia tested. Three patients showed poor fungal killing although the difference from normal for the group as a whole was not statistically significant. The metabolic basis of the impairment in these three patients has not been elucidated

The defective reduction of NBT in chronic granulocytic leukemia confirms previous observations [36] and may be a consequence of the impaired phagocytosis seen in this disease [6 7 26 30] Impaired phagocytosis may lead to a decrease in the metabolic burst which follows phagocytosis and is quantitatively related to the phagocytic load [19] decrease in the metabolic burst may in turn lead to impaired capacity for NBT reduction

There is a suggestion that the decrease in NBT reduction may be greater in Ph[1] positive chronic granulocytic leukemia than in the Ph[1]-negative variety but it will be necessary to study more patients to establish this point

It is now apparent that defective granulocyte function as well as lack of mature neutrophils, may play a role in predisposing patients with leukemia to infection. A spectrum of functional disorders is now emerging, some of which (e.g myeloperoxidase deficiency) are relatively straightforward, but in most cases the metabolic basis remains to be clarified. Further studies of untreated patients are required to elucidate the functional granulocyte disorders in acute and chronic granulocytic leukemia, and to assess their clinical importance.

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Response of Blood and Bone Marrow Neutrophils to the Nitroblue-Tetrazolium Test in Children

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Key Words Bone marrow neutrophils · Endotoxin · Neutrophils · Nitroblue-tetrazolium test

Abstract. The ability of blood and bone marrow neutrophils to reduce nitroblue tetrazolium both before and after stimulation with *Escherichia coli* endotoxin was investigated. The polymorphonuclear cells of the bone marrow are less able to reduce the dye than blood cells. This difference is maintained after stimulation by endotoxin. Early forms of neutrophils (myelocytes and metamyelocytes) have some reducing ability but this is more marked after stimulation with endotoxin.

In recent years numerous observations have emphasized the value of the nitroblue-tetrazolium (NBT) reduction test in the differential diagnosis of bacterial infections. In normal subjects, a positive reaction occurs in 4–14 % of blood neutrophils. During bacterial infection, there is an increased number of NBT-positive neutrophils [2, 5–8, 10]. The reduction of the dye seems to occur more readily when the cell oxidative metabolism is stimulated by bacterial endotoxin [11].

As very little is known about the NBT reduction capacity of the bone marrow phagocytes [3] we have decided to apply this test to blood neutrophils and to bone marrow granulocytic cells simultaneously in order to compare their capacity to reduce NBT.

Materials and Methods

Samples of peripheral blood and bone marrow from 19 children with various diseases were studied. These diseases were: acute lymphoblastic leukemia in remission

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Materials and Methods

Samples of peripheral blood and bone marrow from 19 children with various diseases were studied. These diseases were: acute lymphoblastic leukemia in remission



Fig 1 Myelocytes reducing NBT in bone marrow

2, infectious mononucleosis 1 various viral infections 4, nephrotic syndrome 1 septicemia 1 aplastic anemia 1 sickle cell anemia 1 infection of the urinary tract 3, Mediterranean fever 1 rheumatoid arthritis 2, Wilms tumor 1 and herpetic stomatitis 1

The NBT test was performed according to the method of PARK *et al* [10]. The test was also carried out after the phagocytes had been stimulated *in vitro* with *Escherichia coli* endotoxin [11]. At least 1 000 cells were counted from each sample and the result was expressed as a percentage.

Results

In table I the distribution of NBT scores are shown. The mean percentage of NBT positive blood neutrophils was 9 3% with a range of 0–48% before stimulation and 25.5% with a range of 2–64% after stimulation

For the assessment of the results of the NBT test on the bone marrow cells, the reaction of the immature neutrophils (myelocytes and metamyelocytes) has been considered separately from that of the mature neutrophils (band forms and segmented neutrophils) The mean percentage of the NBT positive immature neutrophils was 0 09% with a range of 0–1% before stimulation and 2.24 % with a range of 0–14 after stimulation The mean percentage of NBT positive mature bone marrow neutrophils was 2% with a range of 0–15% before stimulation, and 8 7% with a range of 0–36% after stimulation

Table I. Percentage of neutrophil cells reducing NBT in blood and bone marrow in various diseases

Case No	Diagnosis	Blood		Bone marrow			
				mature neutrophils		immature neutrophils	
		before stimulation	after stimulation	before stimulation	after stimulation	before stimulation	after stimulation
1	Acute lymphoblastic leukemia	4.5	21	0	1	0	0
2	Acute lymphoblastic leukemia	14	21	0	1	0	0.1
3	Infectious mononucleosis	0	48	0	15	0	4.5
4	Viral infection	9	52	0	0	0	0
5	Viral infection	8	22	0	1	0.2	0
6	Viral infection	2.5	3.5	0	2	0	0
7	Viral infection	3	13	0	2	0.5	0
8	Nephrotic syndrome	3	14	5	11	0	0.5
9	Septicemia	48	64	15	35	0	10
10	Sickle cell anemia	2	8	1	3	0	2
11	Aplastic anemia	7	11.5	6	8	0	0
12	Urinary tract infection	10.5	40	3	8	0	0.5
13	Urinary tract infection	3	8	0	1	0	0
14	Urinary tract infection	19	26		10	1	4
15	Mediterranean fever	7	16	2	22	0	14
16	Rheumatoid arthritis	24	64	4	36	0	7
17	Rheumatoid arthritis	5.5	24	0	2	0	0
18	Wilms' tumor	0.5	2	0	6	0	0
19	Herpetic stomatitis	6	18	0	2	0	0
Mean percentage		9.3	25.5	2	8.7	0.09	2.24

Discussion

The pool of mature nonproliferating bone marrow cells represents approximately 60% of the total phagocytic capacity of the bone marrow cells. Myeloblasts and promyelocytes are not able to phagocytize [12]. Phagocytic activity is attained at the myelocyte to metamyelocyte stage. The polymorphonuclear neutrophils (PMN) have the maximal phagocytic efficiency. Our results show that the NBT reducing capacity of the immature neutrophils is significantly lower than that of mature marrow PMN

It is, however noteworthy that in most cases the reduction of NBT immature marrow PMN was observed after stimulation with endotoxin, implying that under appropriate stimulatory conditions an apparently functionally immature cell can become functionally mature.

McCALL *et al* [9] made the observation that immature myelocytes and promyelocytes do not reduce NBT (perhaps the difference in our results is due to the fact that we used bone marrow cells instead of peripheral cells of patients with acute myelogenous leukemia) Furthermore, we have been able to show that these cells can be stimulated with endotoxin. CATOVSKY and GOLTON [3] also applied the NBT reduction test to immature blood and bone marrow cells in an effort to differentiate acute myelocytic from monocytic leukemia

The great difference between the percentage of positive peripheral and marrow neutrophils was unexpected This difference was statistically significant ($p<0.001$) both before and after stimulation with endotoxin This implies that there may be physiological immaturity of the marrow PMN despite their morphological similarity with the peripheral PMN If this is so it would support the hypothesis that only certain PMN probably those that are functionally mature are sent into circulation

By the use of radioisotopes it has been found that the proportion of blood to bone marrow phagocytes is 100·1 [1 4 12] The control of the production and export into the circulation of marrow PMN is a complex problem which has yet to be fully solved. It appears that besides the mature PMN morphologically immature forms are activated prematurely for phagocytosis and are sent out peripherally in certain situations such as in septicemia [13] For these reasons some metamyelocytes and myelocytes reduce NBT In addition, two of our septicemia cases showed peripheral metamyelocytes with a positive NBT reaction before stimulation with endotoxin and this was probably due to the fact that they had already been stimulated *in vivo* by the infective process.

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Progression and Prognosis of Chronic Myelogenous Leukemia in the Acute Stage Hematologic and Cytogenetic Aspects

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Key Words. Acute transformation of CML Karyotype in leukemia Myelogenous leukemia

Abstract Ten patients with Ph chromosome-positive chronic myelogenous leukemia entering the acute stage were divided hematologically into two different groups. One was characterized by a predominance of myeloblasts in marrow and was cytogenetic either by diploid or hypodiploid, whereas the other had generally low myeloblast counts without significant differences between the peripheral blood and the bone marrow and was characterized by hyperdiploidy It is suggested that an extramedullary acute transformation in the spleen occurs primarily in most cases of the latter group.

Chronic myelogenous leukemia (CML) may be a relatively benign disease. However when acute transformation develops it turns into a rapidly progressive state in most cases [9 13–15] and is one of the most refractory varieties of acute myelogenous leukemia [6] The purpose of the present work is to determine whether clinical hematological and cytogenetical features at the onset of acute transformation could be correlated with the further progression and prognosis of the disease

Materials and Methods

Ten patients with CML entering the acute stage of their disease between 1971 and 1974 were studied hematologically and cytogenetically Karyotypes of these patients in the chronic stage were all diploid with one Ph chromosome except in case 1 [16]. They were all treated mainly with busulfan. The total survival time of these patients was 7 100 months with a mean value of 36.1 months. The diagnosis of acute transformation was made according to the criteria of CANELLOS *et al* [2]

Chromosome preparations of marrow and lymph node cells were made by the direct method, and peripheral leukocytes were cultured without PHA for 24 h [16]. Karyotype analysis of cases, especially with marker chromosomes, was made by the Giemsa banding technique [19].

Results

Hematologic aspects. According to the relative counts of myeloblasts in the peripheral blood and the bone marrow at the diagnosis of acute transformation, these 10 patients were divided into two different groups. The one was characterized by pre dominance of myeloblasts in the bone marrow (cases 1–4), and the other had gener ally few blasts without significant difference in numbers of myeloblasts in the per ipheral blood and the bone marrow. In some of these patients, there were more blasts in the peripheral blood than in the bone marrow (cases 5–10). These two groups are, therefore, identified as the 'blastic' and the 'nonblastic' forms, respec tively. There were no significant differences in hemoglobin levels and thrombocyte counts between these groups.

Clinically cases with the 'nonblastic' form wer characterized by the presence of rapidly progressive splenomegaly or lymph node enlargement and/or lytic bone lesions, whereas in the 'blastic' form splenomegaly was generally not so remarkable (table I).

Cytogenetic aspects. The patients were divided cytogenetically into three groups. Cases 1 and 2 had remained without apparent changes in karyotype until 4 months prior to death when hyperdiploid clones developed and the patients became refrac tory to treatment. In cases 3 and 4, 61 and 76% of marrow cells in metaphase were hypodiploid with 45 chromosomes. Progressive refractoriness to treatment was ac companied by development of new hypo- and pseudodiploid cell lines in case 3. Several kinds of hyperdiploidy ranging from 47 to 54 chromosomes were observed in other patients (cases 5–10) and the incidence of these aneuploid cells ranged from 10 to 23% in the bone marrow and from 20 to 100% in the peripheral blood. However, it w impossible to detect intermediate karyotypes leading to the hyperdiploid stem lines present, except in case 8 in which successive karyotype changes from 47 to 49 chromosomes were observed. In contrast to the number of blast cells and cytogenetic findings in the bone marrow 98 and 100% of lymph node cells from patients 6 and 7 were hyperdiploid atypical myeloblasts containing tw and one Ph chromosomes espectively. Multiple Ph chromosomes were seen in patients 5, 6, 8, and 10 at the diagnosis of acute transformation. The cytogenetic characteristic of the 'blastic' group was either diploidy or hypodiploidy and that of the 'nonblastic' patients was hyperdiploidy at the onset of acute transformation.

All patients were treated with various combinations of two or more drugs consisting of 6-mercaptopurine, methotrexate, vincristine, cyclophosphamide, and prednisolone. Hematologic and cytogenetic remission to CML status was attained once or twice in all cases with the 'blastic form, although the response to treatment was unsatisfactory in cases with

Table I Hematological findings and major clinical signs at the diagnosis of acute transformation

e	Age	Sex	Peripheral blood Hb, g/dl	RBC × 10⁴	WBC × 10⁴	Th × 10⁴	Mbl	Bone marrow Mbl	Spleen size	Lymph node enlarge
	27	M	15.[illegible]	60[illegible]	1 4	4[illegible]0	1	25.2	1 fb	
	16	M	16.0	534	13 6	14 9	45.5	79.6	0	
	55	M	3.[illegible]	9[illegible]	0 16	1 7	12	53.5	4 fb	
	32	M	1[illegible]8	465	5.9	49.5	1[illegible]	61.2	5 fb	
	34	M	13.3	493	3.7	80.9	19	18.8	6 fb	
[illegible]	76	M	11.2	399	2.1	87 8	1.5	4.2	fb	+
	27	M	11 8	433	4.9	86.6	17 0	4 8	5 fb	+
	37	M	16.9	509	4 5	31 6	5 0	5.6	huge	
	[illegible]	M	12.2	383	3.9	5.3	15	14 8	huge	
	4	F	8.5	315	3.7	43 0	17	2.0	huge	

Th = Thrombocytes fb = finger breadth Mbl = myeloblasts.
[1] Case 6 had lytic bone lesion of cervical vertebra.

the 'nonblastic' type except in case 5 in which 15% of bone marrow cells in metaphase was aneuploid with double Ph¹ chromosomes and the survival time was 8 months. The mean survival of patients with the 'blastic' and 'nonblastic' forms was 12 (11–13) and 4 (2–8) months, respectively. There was no clear relationship between the presence of multiple Ph¹ chromosomes and the survival times in the nonblastic group.

Discussion

CML is a clonal disease derived from a hemopoietic stem cell [1] and the acute blast transformation which develops in CML is also suggested to begin in a single stem cell with a Ph¹ chromosome [16]. It is well documented that the appearance of aneuploid clones which have not been observed before in bone marrow cells of a patient with CML, either foreshadows or accompanies the development of acute transformation [18]. Although a few exceptions have been reported [2, 7].

In the present study CML patients at their acute transformation stage were divided in two groups, namely 'blastic' and 'nonblastic'. The presence of blasts or no blasts may depend largely on where the primary focus

of acute transformation is, and further on the characteristics of blast cells developed at that focus. A few cases with probable extramedullary acute transformation other than lienal have been reported [4 8, 10 17 21]. However the spleen must be the major primary focus of extramedullary acute transformation because it is suggested to produce most of the circulating leukemic cells in CML [5] Further among splenectomized patients with CML at the problastic phase, SCHWARZENBERG *et al.* [20] described two cases in which the spleen was infiltrated with blast cells. Significant differences between leukemic cell populations in the bone marrow and the spleen were also documented in one case by cytogenetic study [11] These reports suggest that the bone marrow of most cases with the 'nonblastic' form might be seeded with hyperdiploid blast cells developed during progress of acute transformation in an extramedullary site, especially in the spleen. Based on systematic studies of large specimens from autopsy cases, HASHIMOTO [12] classified the bone marrow of acute transformation into two types, A and B. Type A was characterized by proliferative foci of blast cells in addition to the typical histological picture of CML. The autopsy findings in the bone marrow from patients with the 'nonblastic' form presented here were compatible with Hashimoto s type A and may represent seeded colonies of blast cells derived from an extramedullary site. Certainly there is no evidence other than clinical signs that all cases with the 'blastic' form are of medullary origin and acute transformed blast cells derived from the bone marrow might proliferate in a localized fashion and these could present as the 'nonblastic' type.

Previous cases [2, 3] presented diploidy and hypodiploidy or the 'blastic' form at the time of acute transformation, they had a favorable response to combination chemotherapy while 5 of 6 patients with hyperdiploidy or the 'nonblastic' form did not respond.

The present study shows that hematologic as well as cytogenetic features are valuable in evaluating the mode of progress and the prognosis of CML entering the acute stage.

Acknowledgment. We are indebted to Dr Y HATTA, Kyushu Cancer Center, for permission to study case 10, and doctors in our laboratory for their cooperation in this study

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Table 1 Hematological findings and major clinical signs at the diagnosis of acute transform[illegible]

Case No.	Age	Sex	Peripheral blood					Bone marrow % Mbl	Spleen size	Ly[illegible]
			Hb, g/dl	RBC × 10⁴	WBC × 10⁴	Th × 10⁴	Mbl			
1	27	M	15.[illegible]	602	1 4	4[illegible]0	1	25.2	1 fb	
2	16	M	16.0	534	13.6	14 9	45.5	79 6	0	
3	55	M	3.[illegible]	9	0.16	1 7	12	53.5	4 fb	
4	3	M	12.8	465	5 9	49.5	12	61.2	5 fb	
5	34	M	13.3	493	3.7	80.9	19	18.8	6 fb	
6[1]	6	M	11.[illegible]	399	2.1	87 8	1.5	4.[illegible]	2 fb	+
7	27	M	11.8	433	4 9	86.6	17 0	4 8	5 fb	+
8	37	M	16.9	509	4.5	31 6	5 0	5 6	huge	
9	22	M	1[illegible]2	383	3 9	5.3	15	14.8	large	
10	24	F	8.5	315	3 7	43.0	17	2.0	huge	

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Table I. Sequence differences between the β- and δ-haemoglobin chains of man

Residue No.	Helical No.	β-Chain	δ-Chain
9	A6	Ser	Thr
12	A9	Thr	Asn
22	B4	Glu	Ala
50	D1	Thr	Ser
86	F2	Ala	Ser
87	F3	Thr	Gln
116	G18	His	Arg
117	G19	His	Asn
124/125	H2/H3	Pro	Gln
126	H4	Val	Met

Adapted from reference 5; see also references 1–4.

sideration of homology with many other globin chains [7] leads one to expect that position δ124 would be proline and, therefore, that δ125 would be glutamine. There has however been some uncertainty and either 124 or 125, or both, have been cited as δGln in different textbooks.

Methods

Haemoglobin A_2 was isolated in the carbonmonoxy form from normal individuals by chromatography of haemolysate on DEAE-Sephadex [8]. The purified haemoglobin was converted to globin [9], which was then separated into its component chains by chromatography on CM-cellulose following CLEGG *et al.* [10] except that dithiothreitol was used in place of mercaptoethanol [11] and the pH of the sodium ion gradient was raised in order to separate the δ-chains from any contaminating traces of β-chain [12]. The δ-chains were freed of urea, salts and excess of thiol, freeze-dried, aminoethylated, hydrolysed with trypsin and the tryptic digests 'finger printed' all as previously described [13, 14].

The peptide δTpXIII (δ121–132) was purified from the neutral region of preparative-scale 'fingerprints' by sequential paper electrophoresis at pH 3.5 and pH 9 as described by AMBLER [15]; peptides were located after each stage of purification by staining with fluorescamine [16, 17]. ε-2,4-Dinitrophenol-lysine-HCl and xylene-cyanol FF were used as electrophoresis markers. The δTpXIII was eluted with 0.2 M NH_4OH and dried *in vacuo*. Total acid hydrolysis, determination of the amino acid composition and sequence studies of the peptide were all carried out as previously described [17, 18].

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Residues 124 and 125 (H2 and H3) of the Human Haemoglobin δ-Chain

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Key Words. δ-Chain of haemoglobin Globin chains Haemoglobin structure

Abstract. Residues 124 and 125 of the β-chain of human haemoglobin are prolyl-prolyl. In the δ-chain, one of these positions is occupied by a glutaminyl, and there has been uncertainty as to which of the two residues is the prolyl and which the glutaminyl. The sequence has now been established to be δ124 Pro – 125 Gln.

The haemoglobin of a normal adult comprises about 96–98% Hb A ($\alpha_2\beta_2$) 2–3% Hb A_2 ($\alpha_2\delta_2$) and rather less than 1% Hb F ($\alpha_2\gamma_2$). The β- and δ-chains are very similar and differ at only ten of their 146 amino acid residues. Each of these ten differences, listed in table I has, with one exception, been proved to be correct [1–5] although it has in two cases (positions 50 and 126) been assumed that where an amino acid composition of a peptide indicates a one-to-one substitution such a substitution has indeed occurred. It is worth noting that this is in fact not the case for β/δTpII where a βSer→δThr at position 9 and a βThr→δAsn at position 12 give the overall impression, from amino acid composition alone, of a βSer→δAsn substitution furthermore the fact that Ser→Asn requires two nucleotide changes is no argument against such a substitution since the known difference between β- and δ-chain at position 87 (βThr→δGln) is the result of such a double replacement Hence the nature of position δ50 and δ126 can be accepted with slight reservation It is accepted that one or other of the two prolines found at positions β124 and β125 is replaced in the δ-chain by glutamine [6] but it has not, so far as we are aware been shown which of the two is the site of change. Con-

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sideration of homology with many other globin chains [7] leads one to expect that position δ124 would be proline and, therefore, that δ125 would be glutamine. There has however been some uncertainty and either 124 or 125 or both, have been cited as δGln in different textbooks.

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Abstract. Residues 124 and 125 of the β-chain of human haemoglobin are prolyl-prolyl. In the δ-chain, one of these positions is occupied by a glutaminyl, and there has been uncertainty as to which of the two residues is the prolyl and which the glutaminyl. The sequence has now been established to be δ124 Pro – 125 Gln.

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Table II The amino acid composition of δTpXIII

Amino acid	Molar ratios	Expected for βTpXIII
Thr	0.97 (1)	1
Glu	3.85 (4)	3
Pro	1.13 (1)	2
Ala	2.08 (2)	2
Val	–	1
Met	0.80 (1)	–
Tyr	1.11 (1)	1
Phe	1.05 (1)	1
Lys	1.00 (1)	1

Table III The amino acid sequence of δTpXIII (δ121–132)

Residue No.	121	122	123	124	125	126	127	128	129	130	131	132
Helical No.	GH4	GH5	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
δ-chain	$\overrightarrow{\text{Glu}}$	$\overrightarrow{\text{Phe}}$	$\overrightarrow{\text{Thr}}$	$\overrightarrow{\text{Pro}}$	$\overrightarrow{\text{Gln}}$	Met	Gln	Ala	Ala	Tyr	Gln	Lys
β-chain	Gln	Phe	Thr	Pro	Pro	Val	Gln	Ala	Ala	Tyr	Gln	Lys

$\rightharpoondown$ = The residues identified by sequential dansyl-Edman degradation during this work.

Results and Discussion

The amino acid composition of δTpXIII is shown in table II Compared with βTpXIII it contains one fewer residue each of proline and of valine and one extra residue each of glutamic acid and of methionine. Since δTpXIII is, like βTpXIII neutral at pH 6 4 it can be assumed that the extra residue of glutamic acid has been derived from a glutamine residue which has been converted to glutamic acid during the hydrolysis which precedes amino acid analysis. The N-terminal sequence of δTpXIII (table III) was shown to be 121–125 Glx Phe-Thr Pro-Glx— thus showing quite clearly that the δ-chain has proline at position 124 and glutamine at position 125 We originally had thought that position δ124 (H2) might be of some importance to the function and/or stability of the Hb A_2 molecule since position β124 (H2) forms part of the $\alpha_1\beta_1$ contact in horse oxyhaemoglobin [19] PERUTZ and RAIDT [20] however have made it quite clear that they do not expect this to be the case

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Versuch einer Stadieneinteilung der Osteomyelosklerose nach ferrokinetischen Befunden

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Die Osteomyelosklerose (OMS) zählt zu den myeloproliferativen Syndromen. Der klinische Verlauf ist sehr unterschiedlich und so findet man OMS-Fälle, die ohne nennenswerte Progredienz über 10–15 Jahre verlaufen und solche, die schon nach wenigen Jahren *ad exitum* kommen. Es wird angenommen, dass unter dem Begriff OMS ein uneinheitliches Krankengut subsumiert wird, eine Ansicht, die durch unterschiedliche, ferrokinetische Untersuchungsergebnisse unterstützt wird [1, 3–6, 9–12].

Dennoch durchläuft die OMS mit gewisser Regelmässigkeit einige Krankheitsstadien. Nach einem polyzythämischen Vorstadium werden Zeichen der extramedullären Erythropoese klinisch manifest, worauf allmählich eine Anämie auftritt, bis schliesslich bei exzessiver Leber- und Milzschwellung, Anämie und Thrombopenie der Exitus letalis, entweder durch Komplikationen oder durch einen terminalen leukämischen Schub, erfolgt.

Bei der Polycythaemia vera, die wie die OMS zu den myeloproliferativen Erkrankungen zählt, können auch sehr verschiedene ferrokineti-

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Bei der Polycythaemia vera, die wie die OMS zu den myeloproliferativen Erkrankungen zählt, können auch sehr verschiedene ferrokineti-

sche Untersuchungsergebnisse gefunden werden. POLYCOVE *et al* [10] unterschieden vier distinkte Muster. Ihre «class I» wurde durch erhöhte Erythrozytenproduktion bei normalem Abbau charakterisiert, wogegen bei «class IIa» der gesteigerten Erythrozytenproduktion ein beschleunigter Abbau durch Milzhämolyse, bei «class IIb» durch ineffektive Erythropoese entgegensteht. In «class III» tritt extramedulläre Erythropoese auf. Durch Korrelation mit klinischen Parametern kamen POLYCOVE *et al* zu dem Schluss, dass «class I und IIa» Frühstadien, «class IIb und III» Spätstadien der Erkrankung seien.

Für die OMS wurde ein solcher Einteilungsversuch noch nicht unternommen. In der Annahme, dass auch bei der OMS die differenten ferrokinetischen Muster unterschiedlichen Krankheitsstadien entsprechen könnten, haben wir versucht, unter Berücksichtigung klinischer und ferrokinetischer Befunde eine ähnliche Stadieneinteilung zu treffen.

Patienten

15 Patienten, davon 8 Frauen und 7 Männer mit histologisch gesicherter OMS wurden untersucht und ferrokinetische Daten erhoben. Ein Patient (H. O.) wurde 2mal im Abstand von 7 Jahren untersucht, so dass 16 verwertbare Ergebnisse vorliegen. Die hämatologischen Daten sind in Tabelle I erfasst.

Methodik

15–25 μCi ^{59}FeIII-Citrat wurden intravenös injiziert und innerhalb von 90 min 4mal Blut zur Bestimmung der Plasmaeisenhalbwertszeit (Fe t/2) abgenommen. Der Plasmaeisenumsatz (PET) wurde nach der Formel

$$\frac{\mu g\%\ Fe \times (100-Hk)}{min\ Fe\ t/2 \times 100} = \text{PET in mg/d/100 ml Vollblut}$$

errechnet. Blutabnahmen zur Bestimmung des Eiseneinbaues in die Erythrozyten wurden bis zum 10. Tag vorgenommen. Während dieses Untersuchungszeitraumes wurden auch die Oberflächenmessungen über Leber, Milz und Sakrum durchgeführt. Eine ausführliche Beschreibung der angewendeten Methode findet sich bei HÖFERTZ [4].

Resultate

In Tabelle I sind die hämatologischen Daten und ferrokinetischen Parameter der Patienten erfasst. Die Plasmaeisenhalbwertszeit war in

Tabelle 1. Klinische und hämatologische Befunde

Patient	Stadium	Krankheitsdauer ab Diagnose, Jahre	Hb, g%	Retikulozyten, ‰	Thrombo 1000/µl	Serumbilirubin, mg% gesamt/direkt	Serumeisen, µg%	^{59}Fe t/2, min	Plasmaeisenumsatz, mg Fe/d/100 ml Vollblut	Fe-Einbau in die Erythrozyten, %	Erythropoese in Knochenmark	Erythropoese in Leber	Erythropoese in Milz	Organhämolyse	Ineffektive Erythropoese	Überlebenszeit ab Untersuchung, Monate	Transfusionsbedarf	Häm. Diathese
H.O.L.	I	0,5	17,0	4	400	0,8/0,2	83	41	1,13	92,0	+++	0	0	0	0	85 lebt	0	0
R.F	II	2	14,6			1,2/0,6	98	43	1,24	91,0	+	++	++	0	0	29 lebt	0	0
D.F	II	9	17,0	7	63	1,3/0,3	25	14	0,91		+	++	++	0	0	15 lebt	0	0
P.A.	II	0,2	13,3	26	264	1,1/0,2	66	29	1,30	94,0	++	0	+++	0	0	29 lebt	0	0
H.O.J.L.	II	8	12,4		360	0,9/0,3	49	32	0,98		0	0	+++	0	0	1 lebt	0	0
K.K.	II	1	10,8	18	170	1,2/0,4	72	39	1,27	100	0	++	0	Milz	0	7 lebt	0	0
P.A.	II	8	9,6		252	0,9/0,4	75	42	1,20	78,0	0	0	+++	0	0	12 lebt	0	0
W.E.	II	8	16,5	10	460	4,4/0,4	82	40	1,06	100	++	0	++	Milz	0	28 lebt	0	0
Ch.M.	III	7	9,8	–	17	0,7/0,3	58	55	0,72	54,4	++	0	+++	0	++	12	+	+
H.J	III	13	10,0	20	128	0,9/0,2	45	22	1,43	86,6	+	0	+++	0	+	25 lebt	+	0
K.J.	III	1,5	7,3	–	20	1,0/0,4	28	24	0,90	32,0	0	+	+++	0	++	4	+	+
M.P	III	4	9,5		50	0,9/0,2	93	47	1,47	26,4	0	+	+++	0	+++	19	+	+
M.U.	III	1	8,0	47	28	0,7/0,1	69	25	2,10	47,0	+	+	+++	0	+	6	+	+
R.F	III	3	6,8				58	18	2,50	27,0	0	0	+++	0	++	–	?	+
W.L.	III	2	6,8	12	85	1,2/0,2	114	24	3,80	26,9	+	++	+++	0	++	27 lebt	+	0
W.J	III	8	7,4	4	128	0,9/0,2	115	38	2,32	21,5	+	0	+++	Milz	++	13 lebt	+	+

allen Fällen verkürzt (Normwert 60–100 min) und lag zwischen 14 und 55 min mit einem Mittelwert von 33 4 min. Der Plasmaeisenumsatz war in 15 Fällen erhöht (Normwert 0,38–0 77 mg/d/100 ml Blut) und zeigte Werte zwischen 0 72 und 3 80 mg/d/100 ml Blut. Der Eiseneinbau in die Erythrozyten war in 8 von 14 Fällen pathologisch erniedrigt. Die Oberflächenmessungen zeigten bei 15 Patienten eine extramedulläre Erythropoese, Fall H O I hatte in normales Verteilungsmuster Bei H. O. I. konnte die extramedulläre Erythropoese nur bioptisch und noch nicht ferrokinetisch erfasst werden Über dem Sakrum wurde 1mal ein regelrechter (H. O I) 9mal ein verminderter und 6mal ein fehlender Anstieg registriert. Über der Leber wurde bei 7 Patienten ein primärer Anstieg als Ausdruck der extramedullären Erythropoese gefunden. 14mal war den primäre Anstiege, 1mal ein sekundärer (K. K.) und 1mal kein Anstieg (H. O I) über der Milz gemessen. In 3 Fällen bestanden Hinweise für eine Hämolyse in der Milz (K. K. W E., W I.) Bei 8 Fällen konnte aufgrund des Kurvenverlaufes und der hohen in der Milz verbleibenden Fe Aktivität eine ineffektive Erythropoese festgestellt werden. Von den 8 Patienten mit ineffektiver Erythropoese sind seit Erhebung der ferrokinetischen Daten 4 verstorben von den 8 Patienten mit effektiver Erythropoese sind alle am Leben 7 von 8 Fällen mit ineffektiver Erythropoese mussten in regelmässigen Abständen transfundiert werden alle anderen Patienten waren nicht transfusionsbedürftig.

Wir glauben, dass aufgrund der vorgelegten Ergebnisse 3 ferrokinetisch determinierbare Stadien angenommen werden können (Tab. II): Das Stadium I oder Vorstadium ist bei histologisch gestellter Diagnose durch eine verkürzte Plasmaeisenhalbwertszeit, erhöhten Plasmaeisenumsatz bei normaler Eisenutilisation und unauffälligem Oberflächenaktivitätsverlauf gekennzeichnet. Im Stadium II bzw in der kompensierten Phase ist eine effektive Erythropoese in Milz und/oder Leber und auch im Sakrum nachweisbar Die Eisenutilisation ist normal Das Stadium III bzw die dekompensierte Phase unterscheidet sich vom Stadium II durch das Auftreten einer ineffektiven Erythropoese und pathologisch erniedrigter Fe-Utilisation

Die den drei Stadien zukommende klinische Symptomatik ist in Tabelle III dargestellt. Im Stadium I sind normale bis erhöhte Werte von Erythrozyten, Thrombozyten und *Haemoglobin* und ein palpabler Milztumor zu finden. Stadium II ist durch normale bis mittelgradig verringerte Erythrozyten und Haemoglobinwerte bis etwa 10 0 g%, normale Thrombozytenzahl, deutliche Leber und Milzschwellung sowie

Tabelle II. Unterscheidung von drei Krankheitsstadien durch ferrokinetische Befunde

Stadium	T 1/2	PIT	Eiseneinbau	Ferrokinetisch faßbare extramedulläre Erythropoese	Ineffektive Erythropoese
I Vorstadium	verkürzt	erhöht	normal	0	0
II Kompensierte Phase	verkürzt	erhöht	normal	+	0
III Dekompensierte Phase	verkürzt	erhöht	vermindert	+	+

Tabelle III. Klinische Symptomatologie und Prognose der drei ferrokinetisch differenzierbaren Stadien

Stadium	Erythrozyten	Hb	Milztumor	Thrombozyten	Transfusionsbedarf	Prognose
I	normal bis erhöht	normal bis erhöht	+	normal bis erhöht	–	günstig
II	normal bis erniedrigt	normal bis erniedrigt	+ + (+)	normal	–	günstig
III	erniedrigt	erniedrigt	+ + +	erniedrigt	+	schlecht

allen Fällen verkürzt (Normwert 60–100 min) und lag zwischen 14 und 55 min mit einem Mittelwert von 33 4 min Der Plasmaeisenumsatz war in 15 Fällen erhöht (Normwert 0,38–0 77 mg/d/100 ml Blut) und zeigte Werte zwischen 0 72 und 3 80 mg/d/100 ml Blut. Der Eiseneinbau in die Erythrozyten war in 8 von 14 Fällen pathologisch erniedrigt. Die Oberflächenmessungen zeigten bei 15 Patienten eine extramedulläre Erythropoese Fall H. O I. hatte in normales Verteilungsmuster Bei H. O. I. konnte die extramedulläre Erythropoese nur bioptisch und noch nicht ferrokinetisch erfasst werden Über dem Sakrum wurde 1mal ein regelrechter (H. O I.), 9mal ein verminderter und 6mal ein fehlender Anstieg registriert. Über der Leber wurde bei 7 Patienten ein primärer Anstieg als Ausdruck der extramedullären Erythropoese gefunden. 14mal wurden primäre Anstiege, 1mal ein sekundärer (K. K.) und 1mal kein Anstieg (H O I) über der Milz gemessen. In 3 Fällen bestanden Hinweise für eine Hämolyse in der Milz (K. K., W E. W I). Bei 8 Fällen konnte aufgrund des Kurvenverlaufes und der hohen in der Milz verbleibenden Fe-Aktivität eine ineffektive Erythropoese festgestellt werden Von den 8 Patienten mit ineffektiver Erythropoese sind seit Erhebung der ferrokinetischen Daten 4 verstorben von den 8 Patienten mit effektiver Erythropoese sind alle am Leben 7 von 8 Fällen mit ineffektiver Erythropoese mussten in regelmässigen Abständen transfundiert werden, alle anderen Patienten waren nicht transfusionsbedürftig.

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poese zurückzuführen ist, kann gefolgert werden, dass beide Patienten von BRUNNER [1] eine ineffektive Erythropoese bekamen. Es entspräche dies einem Übergang von Stadium II in Stadium III.

SCHULZ *et al.* [11] konnten keine Korrelation zwischen ferrokinetischen Befunden und dem Krankheitsstadium finden. Sie legten dabei ihren Untersuchungen die histologische Stadieneinteilung von OECHSLIN [8] der in fibroosteoklastisches, ein osteoidoplastisches und ein osteoplastisches Stadium unterscheidet, zugrunde. OECHSLIN betont aber dass im autoptischen Material alle drei Stadien gleichzeitig gefunden werden können. Die fehlende Beziehung zu klinischen und ferrokinetischen Parametern scheint dadurch hinlänglich erklärt.

Nach unseren Untersuchungen besteht eine positive Korrelation zwischen ineffektiver Erythropoese, pathologischer Eisenutilisation, Anämie, Transfusionsbedarf und Thrombozytopenie. Keine Beziehung hingegen findet sich zwischen ferrokinetischen Befunden und Krankheitsdauer ab Diagnosestellung, was durch die oft langen asymptomatischen Verläufe der OMS erklärbar ist. Wenn man daher auch keine Rückschlüsse auf die bisherige Dauer der Erkrankung ziehen kann, so gestattet es das ferrokinetische Muster doch, gewisse prognostische Schlüsse zu ziehen, denn von unseren 8 Patienten mit ineffektiver Erythropoese im Stadium III verstarben ab Zeitpunkt der ferrokinetischen Untersuchung bereits 4 von jenen im Stadium I und II noch keiner. Der im Stadium III auftretenden Thrombozytopenie und den damit verbundenen Blutungskomplikationen dürfte hier entscheidende Bedeutung zukommen.

Wir glauben, dass die ferrokinetische Charakterisierung des Krankheitsstadiums der OMS der histologischen Einteilung überlegen ist, da sie den klinischen Gegebenheiten eher entspricht. Darüber hinaus bietet sie die Möglichkeit prognostischer Aussagen. Stadium I und II können als günstig bezeichnet werden, während im Stadium III die Prognose eher ungünstig zu stellen ist, da mit grosser Wahrscheinlichkeit eine dauernde Transfusionsbehandlung notwendig sein wird und mit Blutungskomplikationen gerechnet werden muss.

Zusammenfassung

Durch ferrokinetische Untersuchungen können drei Stadien der Osteomyelosklerose definiert werden: Stadium I oder Vorstadium mit verkürzter Plasmaeisen-

fehlenden Transfusionsbedarf gekennzeichnet. Im Stadium III schliesslich sind stark erniedrigte Erythrozyten und Haemoglobinwerte (häufig unter 8 0 g%) Thrombozytopenie, excessive Milzschwellung sowie meist regelmässiger Transfusionsbedarf zu finden

Diskussion

Übereinstimmend werden von allen Autoren bei fast allen Patienten mit OMS eine verkürzte Plasmaeisenhalbwertzeit und ein erhöhter Plasmaeisenumsatz gefunden [1 3 6, 7 9 12] Die Eisenutilisation wurde bei einem Teil der Patienten als normal beim anderen Teil als erniedrigt beschrieben Die Ergebnisse der Oberflächenmessungen liessen beim Grossteil der Patienten auf eine extramedulläre Erythropoese schliessen. SZUR und SMITH [12] beschrieben auch einige Fälle mit unauffälligen Oberflächenaktivitäten bei denen nur eine verkürzte Fe t/2 und ein erhöhter Plasmaeisenumsatz gefunden wurden. Eine ineffektive Erythropoese wurde bei etwa der Hälfte aller Fälle gefunden. Wir konnten, wie dies auch von GELINSKY und MÜLLER [3] beschrieben wurde, eine Ineffektivität der Erythropoese nur in der Milz nachweisen Mässige Verkürzung der Lebensdauer der Erythrozyten wurde bei einem Grossteil der Fälle nachgewiesen [1 3 7 9 12] stärkere organgebundene Haemolysen hingegen scheinen weniger häufig zu sein [6, 7]

Ferrokinetische Nachuntersuchungen von Patienten wurden von SZUR und SMITH [12] BRUNNER [1] und von uns durchgeführt. Einer der von SZUR und SMITH beschriebenen Fälle mit unauffälligen Oberflächenaktivitäten und unsere Patientin H. O I zeigten einige Jahre später eine massive extramedulläre Erythropoese in der Milz. Bei Patientin H. O I. wurde ausserdem eine beträchtliche Vergrösserung des Milztumors und ein Absinken der Hb-Werte von 17 0 auf 13 4 g% gefunden Dies entspräche einem Übergang von Stadium I in Stadium II BRUNNER [1] berichtet über zwei Fälle, bei denen die Oberflächenmessungen zunächst auf extramedulläre und auch medulläre Erythropoese schliessen liessen, die aber bei der zweiten Untersuchung ausschliesslich extramedullär Blut bildeten und darüber hinaus ein deutliches Absinken der Eisenutilisation in den pathologischen Bereich zeigten Da nun die pathologische Eisenutilisation nach den Ergebnissen von SZUR und SMITH [12] GELINSKY und MÜLLER [3] und auch unseren Untersuchungen bei OMS nicht auf aplastische Störungen sondern stets auf die Ineffektivität der Erythro-

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Platelet and Leukocyte Antibodies in Autoimmune Hemolytic Anemia

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Key Words. Autoantibodies · Erythrocyte antibodies · Granulocytopenia · Hemolytic anemia · Leukocyte antibodies · Platelet antibodies · Thrombocytopenia

Abstract. Platelet and leukocyte counts and leukocytotoxic and platelet antibodies were studied in 32 patients with autoimmune hemolytic anemia (AHA). Leukopenia was present in 59.4%, thrombocytopenia in 59.4% and leuko-thrombocytopenia in 40.5% of the cases. Specific antibodies for granulocytes were found in 81.3%, platelet antibodies in 90.6%. The AHA, leukopenia and thrombocytopenia generally presented dissociated evolution and different response to immunosuppressive treatment. The leukopenia of 2 and the thrombocytopenia of 6 patients appeared at variable time intervals after the AHA or the detection of leukocyte and platelet antibodies. Thrombocytopenic purpura was present in 6 patients, and in 2 of these since infancy. AHA may thus be complex autoimmune syndrome that may involve leukocytes and platelets as well as erythrocytes, with synthesis of autoantibodies specific for different blood cells.

In patients with autoimmune hemolytic anemia (AHA) the number of leukocytes and platelets is generally not a subject of particular study nor are leukocyte and platelet antibodies systematically sought. Nevertheless, cases of leukopenia at times associated with leukocyte antibodies have been reported in a few instances [2, 4, 5, 9, 17, 20]. Allgood and Chaplin [1] reported leukopenia in 13% of the cases. Better described is the thrombocytopenia, at times so severe as to produce hemorrhagic purpura, its association with AHA being known as Evans' syndrome [8]. The incidence of thrombocytopenia in AHA is 13.2% according to Dausset and Colombani [6], 8.3% according to Brunet et al. [5], considerably lower (1.6%) in the series reported by Silverstein and Heck [21]. Pirofsky [18] reported 5 cases of pancytopenia and 4 of Evans' syndrome out of

halbwertszeit, erhöhtem Plasmaeisenumsatz und unauffälligen Oberflächenaktivitäten Stadium II oder kompensiertes Stadium mit ferrokinetisch fassbarer extramedullärer Erythropoese und normaler Eisenutilisation und Stadium III oder dekompensiertes Stadium mit ineffektiver Erythropoese. Diese Einteilung scheint den klinischen Gegebenheiten besser zu entsprechen als die histologische Klassifizierung und scheint ausserdem einige prognostische Relevanz zu haben.

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Platelet and Leukocyte Antibodies in Autoimmune Hemolytic Anemia

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Key Words. Autoantibodies · Erythrocyte antibodies · Granulocytopenia · Hemolytic anemia · Leukocyte antibodies · Platelet antibodies · Thrombocytopenia

Abstract. Platelet and leukocyte counts and leukocytotoxic and platelet antibodies were studied in 32 patients with autoimmune hemolytic anemia (AHA). Leukopenia was present in 59.4%, thrombocytopenia in 59.4% and leuko-thrombocytopenia in 40.5% of the cases. Specific antibodies for granulocytes were found in 81.3%, platelet antibodies in 90.6%. The AHA, leukopenia and thrombocytopenia generally presented a dissociated evolution and a different response to immunosuppressive treatment. The leukopenia of 2 and the thrombocytopenia of 6 patients appeared at variable time intervals after the AHA or the detection of leukocyte and platelet antibodies. Thrombocytopenic purpura was present in 6 patients, and in 2 of these since infancy. AHA may thus be a complex autoimmune syndrome that may involve leukocytes and platelets as well as erythrocytes, with synthesis of autoantibodies specific for different blood cells.

In patients with autoimmune hemolytic anemia (AHA) the number of leukocytes and platelets is generally not a subject of particular study nor are leukocyte and platelet antibodies systematically sought. Nevertheless, cases of leukopenia at times associated with leukocyte antibodies have been reported in a few instances [2, 4, 5, 9, 17, 20]. Algood and Chaplin [1] reported leukopenia in 13% of the cases. Better described is the thrombocytopenia, at times so severe as to produce hemorrhagic purpura, its association with AHA being known as Evans' syndrome [8]. The incidence of thrombocytopenia in AHA is 13.2% according to Dausset and Colombani [6], 8.3% according to Brunet *et al.* [5], considerably lower (1.6%) in the series reported by Silverstein and Heck [21]. Pirofsky [18] reported 5 cases of pancytopenia and 4 of Evans' syndrome out of

137 cases of AHA. Seldom platelet antibodies have been found [3, 5 9, 12, 15 19]

These observations are in favor of the hypothesis that leukopenia and thrombocytopenia during AHA could be explained, as the hemolytic anemia, on an autoimmune pathogenesis. We herein present the results of a study on the quantitative variations of leukocytes and platelets during the various stages of the disease, and leukocyte and platelet antibodies in 32 patients with AHA, either isolated or during the course of non-neoplastic diseases.

Materials and Methods

32 patients with AHA were studied, 27 were isolated cases, whereas in the remaining 5 patients the hemolytic syndrome appeared during chronic active hepatitis, systemic lupus erythematosus, Hashimoto s thyroiditis, thrombotic thrombocytopenic purpura and liver cirrhosis. The patients were examined during the initial stages of the hemolytic syndrome and later controlled during remission or relapse. No patients received immunosuppressive therapy Only case No. 25 received blood transfusions. The erythrocyte and leukocyte counts were made with the Coulter Counter FN the platelet count with the Autoanalyzer Technicon 1A. Patients were considered leukopenic when the leukocyte count was ≦4,000/μl, and thrombopenic when the platelets were ≦140,000 μl. Erythrocyte antibodies were studied by agglutination in saline, by the direct Coombs' test with anti-γ-globulin and specific anti-IgG, anti-IgA, anti-IgM and anti-complement sera [11], by the indirect Coombs' test, by agglutination with enzyme-treated cells and hemolysin assay Leukocyte antibodies were demonstrated by the cytotoxic test [7] testing six samples of allogenic leukocytes typed for the antigens of the HLA system. Platelet antibodies were shown by the thromboagglutination and indirect antiglobulin consumption test (AGCT) according to an already described method [10] with anti-γ-globulin, anti-IgG and anti-IgM sera, for the definition of the immunoglobulin type the incomplete antibodies were fixed to target platelets. The cytotoxic and thromboagglutination tests were also performed with eluates of the Coombs-positive erythrocytes. The need for blood transfusions in numerous patients did not allow a systematic research of leukocyte and platelet antibodies in the later stages of the disease.

Results

The results are shown in table I In table II the data regarding the incidence of leukopenia and/or thrombocytopenia and leukocyte and platelet antibodies are listed. The leukopenia was in all cases due to neutropenia, only in one case (No. 28) there was also lymphopenia. In all patients, except case No 27 with severe myeloid hypoplasia, the bone mar

Table I. Clinical and [illegible]oratory data in [illegible] cases w[illegible] [illegible] common [illegible]

Case No.	Sex	Age years	Leuko-penia	Thrombo-cytopenia	Leuko-cytotoxi-city	Platelet antibodies AGCT	Platelet antibodies aggl.	Erythrocyte autoantibodies	Diagnosis* and evolution
1	F	27	+	–	+	+	+	IgA	AHA and leukopenia responsive to steroids
2	F	51	+	–	+	–	–	IgG+IgA+IgM+C'	CAH; AHA unresponsive, leukopenia responsive to steroids
3	F	23	+	+	+	+	–	IgG	TP from early age splenomegaly; AHA and leukopenia responsive to steroids remission of thrombocytopenia after splenectomy
4	F	55	+	–	+	–	+	IgG+IgA+IgM+C'	AHA and leukopenia responsive to steroids
5	F	66	+	+	+	+	+	IgG+IgA+IgM+C'	AHA and leuko-[illegible] un-responsive to steroids; death after 6 months
6	F	70	–	–	–	+	–	IgA+IgM+C'	–
7	M	58	–	–	–	+	–	IgG	–
8	F	30	+	+	+	+	–	IgG+IgA	splenomegaly pancytopenia unresponsive to steroids remission of leuko-thrombocyto-penia after splenectomy; persisting AHA
9	F	37	+	+	+	+	–	IgA+C'	AHA and leukopenia responsive to steroids unresponsive thrombocytopenia
10	F	23	–	+	+	+	+	IgG	SLE, TP during remission of AHA respon-sive to steroids
11	F	33	–	–	+	+	–	IgA+C'	
12	M	[illegible]	+	–	+	+	–	IgM+C'	spontaneous remission of AHA; persisting leukopenia

Table 1 (continued)

Case No.	Sex	Age years	Leuko-penia	Thrombo-cytopenia	Leuko-cytotoxi-city	Platelet antibodies		Erythrocyte autoantibodies	Diagnosis[a] and evolution
						AGCT	aggl.		
13	M	60	–	+	+	+	–	IgG	thrombocytopenia responsive to steroids after 2 months of AHA
14	F	17	–	–	–	+	–	IgG+IgA+IgM+C′	–
15	F	55	+	+	+	–	–	IgA+IgM+C′	AHA and leuko-thrombocytopenia unresponsive to steroids
16	F	66	+	+	+	+	+	IgA+IgM+C′	AHA and leuko-thrombocytopenia unresponsive to steroids
17	F	76	–	–	–	+	–	IgG	–
18	F	58	–	–	+	–	+	IgG+IgM+C′	–
19	F	40	+	+	+	–	+	IgG+IgA+C′	chronic leuko-thrombocytopenia revealed after 5 years of AHA
20	F	55	+	+	–	+	+	IgG	recurrent thrombocytopenia after 2 years of AHA steroids and Imuran partially effective on leuko-thrombocytopenia
21	F	58	+	–	+	+	+	IgG+IgA+C′	steroids and Imuran partially effective on AHA, ineffective of leuko-thrombocytopenia
22	F	26	–	+	+	+	–	IgG+IgA+IgM+C′	not treated
23	M	69	+	–	+	–	+	IgG+IgA+IgM	–
24	F	55	–	+	+	+	+	IgG	thrombocytopenia after 2 years of AHA both partially responsive to steroids

Table I (continued)

Case No.	Sex	Age years	Leuko-penia	Thrombo-cytopenia	Leuko-cytotoxicity	Platelet antibodies		Erythrocyte autoantibodies	Diagnosis[a] and evolution
						AGCT	aggl.		
25	F	35	–	+	+	+	+	IgG + IgM + C′	TP from early age- AHA responsive to steroids remission of thrombocytopenia after splenectomy
26	F	1	–	+	+	+	+	IgG + IgA + IgM + C′	TP responsive to steroids and cyclophosphamide, AHA unresponsive
27	F	71	+	+	+	+	+	IgA + IgM + C′	TP not treated
28	F	36	+	+	–	+	+	IgG	HT· AHA responsive to steroids TP after 1 ½ years of AHA leuko-thrombocytopenia responsive to splenectomy
29	M	43	+	+	+	+	+	C′	AHA and leuko-thrombocytopenia unresponsive to steroids
30	M	35	+	+	+	+	+	IgA + C′	TTP unresponsive to steroids; death after 1 month
31	F	58	–	–	+	–	–	IgG	LC AHA responsive to steroids leukopenia after 4 months of AHA
32	F	24	+	+	+	–	+	C′	AHA in pregnancy

CAH = Chronic active hepatitis TP = thrombocytopenic purpura HT = Hashimoto's thyroiditis TTP = thrombotic thrombocytopenic purpura LC = liver cirrhosis.

[a] 3 months.

Table II Incidence of leukopenia, thrombocytopenia, leukocyte and platelet antibodies in 32 patients with autoimmune hemolytic anemia (AHA)

	Number of cases	%
Leukopenia	19	59.4
Thrombocytopenia	19	59.4
Leuko-thrombocytopenia	13	40.6
Thrombocytopenia before AHA	2	6.3
Thrombocytopenia after AHA	6	18.8
Leukopenia after AHA	2	6.3
Thrombocytopenic purpura	6	18.8
Leukocytoxicity	26	81.3
Platelet antibodies (aggl. and/or AGCT)	29	90.6
Leukocyte + platelet antibodies	23	71.9

row showed a normal or increased number of cells with erythroblastic hyperplasia. In the thrombocytopenic patients the megakaryocytes, either in normal or increased number presented regressive alterations and, in particular the cytoplasma in the granular stage without platelet formation. In two cases (No 13 15) a severe reduction of megakaryocytes was evident. The granulocytic series was generally normal and only a few leukopenic patients presented a reduction of the mature granulocytes with an increase of myelocytes and promyelocytes.

The autoantibodies fixed to the erythrocytes were of a different immunoglobulin type in each case, whether or not they fixed the complement. A raised titer of cold agglutinins (1/128 – 1/32,000) was found in the serum of patients No 6, 18 21 25 29 In all cases, the leukotoxic antibodies were directed against the granulocytes and in only three cases (No. 9 22, 24) also against lymphocytes. Each serum contained antibodies directed against a different number of leukocyte samples (1–6) without it being possible to statistically show a specificity with particular HLA antigens. Even the thromboagglutination test resulted positive against a diverse number of platelet samples in the single cases. The indirect AGCT on platelets was positive for anti-γ-globulin and anti IgG sera, negative for anti-IgM serum. The positivity of the immunologic tests was found only in initial stages of the hemolytic syndrome only in case No 28 the indirect AGCT on platelets and thromboagglutination became positive,

together with the appearance of thrombocytopenia after 2 years, when the AHA was in a phase of therapeutic remission. Whereas in about one third of the patients the finding of leukocyte and/or platelet antibodies was not associated or followed by leukopenia and/or thrombocytopenia, in only one case (No. 20) of leukopenia and in one of thrombocytopenia (No. 15) no corresponding antibodies were demonstrable. A relationship was not found between the presence of leukocyte and/or platelet antibodies and the immunochemical type of the erythrocyte autoantibodies. The tests of leukocytotoxicity and thromboagglutination carried out on Coombs'-positive erythrocyte eluates were negative in all cases.

Immunosuppressive therapy with steroids, at times associated with Imuran or cyclophosphamide, had a variable effect in each patient and often also towards the various types of hemocytopenia in the single patient. As the therapy was effective on the AHA, it did not prevent the appearance of leukopenia or thrombocytopenia. On the contrary as the therapy proved ineffective towards the AHA, it was effective on the leukopenia or thrombocytopenia. Leukopenia was relatively sensitive to therapy showing a remission in 6 cases, an unstable response in 1 case, and no effect in 7 cases, 3 of which benefited from splenectomy. Thrombocytopenia, on the other hand, was resistant to immunosuppressive agents: a remission was achieved in only 3 patients, a partial and temporary effect in 2, and no effect in 11 cases, in 4 of which splenectomy brought about a return to normal of the platelet count. Cases of leukothrombocytopenia were very resistant to therapy and had an unfavorable prognosis.

Discussion

The high incidence of leukopenia and/or thrombocytopenia, and of leukocyte and platelet antibodies in patients with AHA, indicates that this syndrome represents a complex immunohematologic disorder not only limited to erythrocytes but involving also the other types of blood cells. A characteristic of this syndrome is also the evolution, often dissociated, of the single types of hemocytopenia with considerable variety of the hematologic pictures and the different response to immunosuppressive therapy. Two points are to be discussed: the pathogenetic role of the leukocyte and platelet antibodies producing leukopenia and thrombocytopenia and their serologic relationship with the erythrocyte autoimmune process. The presence of cytotoxic antibodies against granulocytes and complete and in-

complete platelet antibodies in leukopenic and thrombocytopenic patients without blood transfusions or with numerous pregnancies could be referred to an autoimmune process against granulocytes and platelets. The negative leukocytotoxic and thromboagglutination test with erythrocytes eluates and the independent presence in some patients of erythrocyte, leukocyte and platelet antibodies exclude a nonspecific fixation to the cells. The positive AGCT on platelets with anti-γ-globulin and anti IgG sera and the negative reaction with anti-IgM serum indicate that the incomplete platelet antibodies are of the IgG-type.

The incidence of leukopenia and/or thrombocytopenia found in our cases is higher than that reported by others. This can be explained, at least in part by our prolonged observation of the patients which showed, even after years of AHA, leukopenia and/or thrombocytopenia. This fact demonstrates also the pathogenetic importance of the antibodies and explains their presence in patients with normal leukocyte and platelet counts. There is, therefore, an interval of time between the synthesis of the autoantibodies and the appearance of cytopenia, which may depend on various factors entity and duration of the antibody sensitization and cellular destruction as well as compensatory capacity of the bone marrow These varieties could also explain the chronologically dissociated courses and diverse therapeutic responses, in the same patient, of the hemolytic anemia, leukopenia and thrombocytopenia The bone marrow has shown morphologic changes of various severity These alterations also depend on the selective fixation of the autoantibodies to the bone marrow cells, which has been serologically demonstrated by direct AGCT [3 22] or immunofluorescence [16] whereas the dynamics have been studied by injection of heterologous antisera marked with fluorescein or ^{131}I [13 14] Other cytopenic factors, such as splenic hypersequestration may nevertheless intervene. In patients with splenomegaly leukopenia and/or thrombocytopenia resistant to immunosuppressive therapy have been corrected by splenectomy

AHA may thus be considered as a complex autoimmune syndrome which involved the erythrocyte, granulocyte and platelet series during different stages of the disease by specific autoantibodies for each cell type. Although patients with autoimmune disorders are prone to develop various autoimmune phenomena, their considerable frequency during AHA is not explained It may be an immune disorder which causes the loss of immunologic tolerance for cells, as those of the blood which originate from the same stem cell.

From a practical point of view it is important to follow AHA patients, as they may manifest thrombocytopenic purpura or pancytopenia. It is also fundamental to systematically search for antibodies against leukocytes, platelets as well as erythrocytes.

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Colony Formation by Canine Hemopoietic Cells *in vitro*

Inhibition by Polymorphonuclear Leukocytes[1]

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Key Words. Bone marrow culture · Canine leukocytes · Cell culture · Colony formation · Polymorphonuclear leukocytes

Abstract. In soft agar cultures of canine blood leukocytes, an inhibition of colony formation was observed relative to the size of the inoculum. Analysis of the cellular composition of the inoculum suggested that this inhibition was associated with the number of polymorphonuclear leukocytes present. Removal of phagocytic cells by the iron ingestion method or selective destruction of granulocytes by freezing in the presence of dimethyl sulfoxide eliminated the inhibitory action on colony formation. In mixed cultures of canine bone marrow and autologous blood leukocytes, a similar inhibition of colony formation was observed. The results presented indicate that polymorphonuclear leukocytes, if present in a concentration exceeding 2.5×10^6/ml of inoculum, inhibit *in vitro* granulocytic/monocytic colony formation.

In vitro growth of granulocytic and/or macrophage colonies in soft agar cultures [1–10] is influenced, in part, by the interaction of cells being plated. Such interactions may enhance or inhibit colony formation in cultures of human bone marrow cells [7]. It has also been reported that macrophages and granulocytes inhibit colony formation by mouse and rat hemopoietic stem cells [8, 9, 11]. Our present experiments provide evidence that colony formation by canine peripheral blood leukocytes and bone marrow cells is inhibited when excessive number of polymorphonuclear leukocytes (PMN) are present in the culture.

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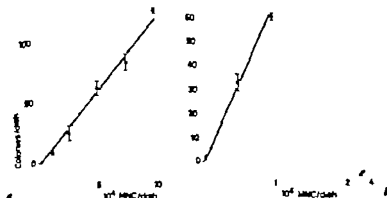


Fig. 1. Number of colonies per dish plotted against the number of mononuclear cells (MNC) inoculated. Vertical bars indicate ±1 SE. *a* In cultures of bone marrow cells. *b* In cultures of blood leukocytes.

experiment is presented in figure 1b, where the inoculum response curve seemed to remain linear up to 1×10^6 mononuclear cells per dish (forming about 60 colonies), but no colony formation was observed in plates containing 2×10^6 or 4×10^6 mononuclear cells. The ratio of PMN to mononuclear cells was 2.4 in this experiment. In terms of the number of PMN accompanying the mononuclear cells, the inoculum response curve was linear until 2.4×10^6 PMN per inoculum were present no colony formation occurred when 4.8×10^6 and 9.6×10^6 PMN were present.

As the above phenomenon might be explicable in terms of an inhibition by PMN present in high numbers, attempts were made to correlate colony formation with varying numbers of PMN in cultures containing a constant number (0.5×10^6) of mononuclear blood leukocytes. The results from 85 cultures are presented in figure 2. In the 75 cultures showing colony formation, the number of accompanying PMN per dish never exceeded 3.5×10^6 and only in five cases was higher than 2.5×10^6. In all of the remaining ten cultures showing no colony formation, the inoculum contained more than 2.5×10^6 and in seven of these cultures even more than 3.5×10^6 PMN.

Figure 3 shows the results of 18 inoculum response experiments with blood leukocytes. As the plating efficiency was not uniform for the various experiments, the number of colonies was expressed relative to those formed with 0.5×10^6 mononuclear leukocytes in each experiment. The

Materials and Methods

Beagles of both sexes were used as bone marrow cell and blood leukocyte donors. Bone marrow was aspirated from the iliac crest and anticoagulated with EDTA. After sedimentation with dextran (molec. wt. approximately 250,000) the nucleated-cell rich plasma was removed and centrifuged. The cells were washed and the mononuclear and polymorphonuclear cells counted differentially in a Neubauer chamber at 500×magnification. Venous blood was anticoagulated with heparin and the leukocytes were isolated by dextran sedimentation, then washed and counted differentially as for the bone marrow cells. In some of the experiments, leukocytes were collected from peripheral blood by leukapheresis using an NCI IBM experimental blood cell separator as described elsewhere [10].

Cultures were set up in MEM for Spinner cultures (Gibco), supplemented with vitamins, sodium pyruvate, amino acids and 20% horse serum (Flow Labs); then agar was added to a final concentration of 0.3%, as described in detail elsewhere [10]. Various numbers of cells were plated in triplicate or quadruplicate in 35-mm plastic Petri dishes (1 ml medium in each). As PMN are generally considered unable to proliferate, the size of the inoculum was given in terms of mononuclear cells, though the number of accompanying PMN was also determined and recorded. Serum from dogs killed 10 days after 1,200 rad whole body X-irradiation served as the source of colony stimulating activity and was added to each dish before plating in amounts of 0.3 and 0.2 ml for bone marrow and blood leukocyte cultures, respectively. These quantities of serum supported maximal growth of colonies, i.e., increased amounts failed to increase the number of colonies. Cultures were incubated in desiccators containing 3% CO_2 in humidified air according to FIRKET [5], for 7 days at 37 °C. Colonies were defined as aggregates of at least 15 cells and were counted under a dissecting microscope at 30×magnification.

For the removal of phagocytes, cells were suspended in MEM and colloidal iron carbonyl was added. After incubation for 30 min at 37 °C, cells that had ingested iron were removed by means of a magnet outside the tube. For the selective destruction of granulocytes, cells were suspended in MEM containing 10% dimethyl sulfoxide, then frozen and thawed, as described elsewhere [2].

Result

The number of colonies formed in cultures of bone marrow cells was found to be a linear function of the number of cells inoculated (mononuclear) within broad limits (fig. 1a). In bone marrow cultures, no sign of inhibition of colony growth could be observed even in plates containing as many as 1 000 colonies. On the other hand in cultures of blood leukocytes, an increase in inoculum size above certain limits failed to increase the number of colonies. In fact, if the size of the inoculum was too large, it resulted in a complete lack of colony formation. Such an

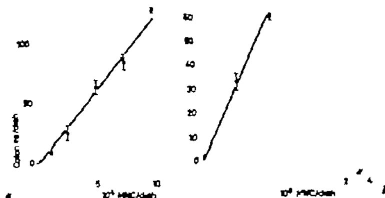


Fig. 1. Number of colonies per dish plotted against the number of mononuclear cells (MNC) inoculated. Vertical bars indicate ±1 SE. *a* In cultures of bone marrow cells. *b* In cultures of blood leukocytes.

experiment is presented in figure 1b, where the inoculum response curve seemed to remain linear up to 1×10^6 mononuclear cells per dish (forming about 60 colonies), but no colony formation was observed in plates containing 2×10^6 or 4×10^6 mononuclear cells. The ratio of PMN to mononuclear cells was 2.4 in this experiment. In terms of the number of PMN accompanying the mononuclear cells, the inoculum response curve was linear until 2.4×10^6 PMN per inoculum were present no colony formation occurred when 4.8×10^6 and 9.6×10^6 PMN were present.

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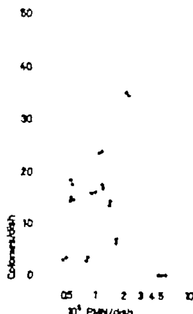


Fig 2 Number of colonies per dish plotted against the number of PMN per dish in cultures set up from various blood leukocyte samples. In each culture 0.5×10^4 MNC were plated accompanied by varying numbers of PMN as indicated on the abscissa.

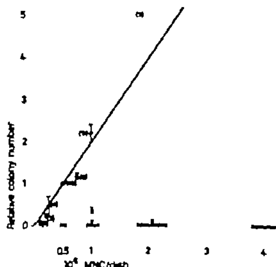


Fig 3 Relative colony number in cultures containing less (● = mean±SE, number in parenthesis indicates the number of separate experiments) and more (x = results of separate experiments) than 3×10^4 PMN plotted against the number of MNC plated. Colony count in cultures inoculated with 0.5×10^4 MNC was taken as the unit in each experiment.

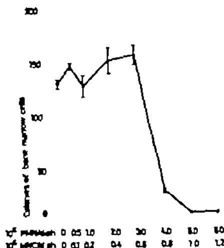


Fig. 4. Number of colonies of bone marrow origin in mixed cultures of a constant inoculum of bone marrow containing 10^5 MNC and of varying numbers of leukocytes as indicated on the abscissa. Mean ± SE.

counts of cultures which contained more than 3×10^6 PMN are by symbols different than those showing the number of colonies in cultures with less than 3×10^6 PMN. In most cultures with more 3×10^6 PMN, no colonies grew; in the few cultures that showed colony formation, the number of colonies was always below the linear derived from the colony counts of cultures with less than 10^6 PMN.

Four million mononuclear blood leukocytes in a culture dish usually high numbers of PMN and colonies failed to form. Growth of colonies was observed, however, when the same number of lear leukocytes from the same cell suspension was plated after of the phagocytic cells by iron carbonyl or after selective inactiof granulocytes by freezing and thawing (table I).

To see whether colony formation by bone marrow cells was similarly by the presence of high numbers of PMN, a constant number dog bone marrow mononuclear cells (10^5) was plated together with creasing numbers of autologous blood leukocytes. The small number colonies formed in cultures inoculated with an identical number of leukocytes without any bone marrow cells, was subtracted from the



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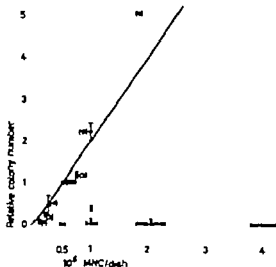


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Four million mononuclear blood leukocytes in a culture dish usually meant high numbers of PMN and colonies failed to form. Growth of numerous colonies was observed, however, when the same number of mononuclear leukocytes from the same cell suspension was plated after removal of the phagocytic cells by iron carbonyl or after selective inactivation of granulocytes by freezing and thawing (table I).

To see whether colony formation by bone marrow cells was similarly inhibited by the presence of high numbers of PMN, a constant number of dog bone marrow mononuclear cells (10^6) was plated together with increasing numbers of autologous blood leukocytes. The small number of colonies formed in cultures inoculated with an identical number of blood leukocytes without any bone marrow cells, was subtracted from the

Table I Effect of the removal of phagocytes or of freezing and thawing on the number of colonies in cultures of blood leukocytes

Treatment of leukocytes before plating	Million cells plated per dish		Number of dishes	Colonies per dish (mean ± SE)
	mono-nuclear	polymorpho-nuclear		
No treatment	4.0	7.1	3	0
Removal of phagocytes	4.0	0.3	3	176 ± 10
Freezing and thawing	4.0	destructed	2	362 ± 12

colony count of the corresponding mixed culture in order to determine the net number of colonies of bone marrow origin. The number of bone marrow colonies was not reduced by the presence of less than 3×10^4 PMN in a culture dish. However, colony formation was substantially diminished in cultures containing 4×10^4 PMN; colonies failed to grow with more than 4×10^4 PMN per dish (fig. 4).

Discussion

As peripheral blood leukocytes of normal dogs contain only about 15 *in vitro* colony forming units (CFU) per 10^6 cells [10], culture inocula with one to three million leukocytes produced, in some cases, too few colonies to allow reliable quantification of CFU in peripheral blood. In order to improve the accuracy of the estimation it would have been necessary to increase the number of colonies counted. A simple solution to this problem could have been to increase the inoculum size in such a way the number of colonies per plate in cultures of canine bone marrow could have been increased far above those easy to count. An increase in the inoculum size above certain limits in cultures of canine peripheral blood leukocytes, however, resulted in a complete inhibition of colony formation. This phenomenon might be explained by hypothesizing the presence of an inhibitory cell among blood leukocytes, the effect of which is manifest when the number of such cells in the culture exceeds a critical value. By increasing the inoculum size, this critical value may be reached, thereby resulting in inhibition of colony formation.

As for the identity of this postulated inhibitory cell, PMN have been reported to inhibit colony formation in other species [3, 7–9, 11].

Therefore, the question arises, whether the lack of colony formation in some canine leukocyte cultures might be related to the number of PMN present in the inoculum. In cultures inoculated with a constant number (0.5×10^6) of mononuclear leukocytes, some colonies always grew when the number of accompanying PMN did not exceed 2.5×10^6 while no colony formation occurred in cultures containing more than 3.5×10^6 PMN. This suggests that the absence of colony formation may indeed be associated with the presence of big numbers of PMN in a culture and that the critical number for inhibition lies between 2.5 and 3.5×10^6 PMN per dish (for a 1.2-ml culture). Data presented in figure 3 show that, within each experiment, the plating efficiency (number of colonies per mononuclear cell plated) of leukocyte cultures was independent of the inoculum size in plates containing less than 3×10^6 PMN. It was greatly diminished in cultures with more than 3×10^6 PMN. This supports the view that the lack of colony formation observed with higher inoculum size in cultures of peripheral blood leukocytes may be related to the high number of PMN present.

In plates containing 4×10^6 mononuclear cells with 7×10^6 accompanying PMN no colonies grew at all. However numerous colonies were formed from the same number of mononuclear leukocytes from the same leukocyte suspension when the number of accompanying PMN was substantially reduced, either by removal of phagocytes or by selective destruction of PMN by freezing before plating. This observation provides direct evidence for the assumption that the presence of high numbers of PMN in a culture inhibits colony formation.

The lack of such a phenomenon in cultures of canine bone marrow cells may be explained as follows. The frequency of CFU in canine bone marrow cells is about 100 times higher than in canine peripheral blood leukocytes. For this reason, increasing the inoculum size results in too high a number of colonies in a bone marrow culture to be counted; this occurs with an inoculum cell count far below that containing a critical number of PMN. Data presented in figure 4 however show that colony formation by canine bone marrow cells is definitely susceptible to the inhibition by the presence of high numbers of added blood PMN.

The above inhibitory effect of PMN may be explained (a) by assuming that PMN consume a component in the medium necessary for colony formation, or (b) by assuming that canine PMN contain and/or produce a factor that inhibits colony formation. Other experiments by our group have shown that a medium conditioned by high numbers of can

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ine leukocytes inhibits colony formation [3] and CHERVENICK and LO-BUGLIO [4] have observed a similar effect with a medium conditioned by human granulocytes these results support the second assumption

Our observations reveal a possible pitfall in the estimation of CFU in canine peripheral blood On the basis of our results, cultures showing no colonies cannot be used as evidence for the lack of CFU in the leukocytes plated without stating that the concentration of PMN in the culture did not exceed the threshold value for inhibition. They also raise questions about the nature of the factor produced by polymorphonuclear cells that is capable of inhibiting proliferation of granulocytic precursor cells and about its physiological role, if any in regulating granulocytic production by inhibitory mechanisms, as has been recently postulated [6]

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Materials and Methods

During the genetic counselling for the G-6-PD deficiency carried out in the Ferrara district, blood samples from 2,500 schoolboys were collected in heparinized capillary tubes for screening of G-6-PD activity.

The dye decolorization test of MOTULSKY and CAMPBELL-KRAUT was performed and interpreted according to the recommendations of the WHO technical report for the study of G-6-PD [16]. In all the cases showing decrease of the decolorization rate, an assay of G-6-PD activity of the red blood cells and an evaluation of the electrophoretic mobility of the enzyme were carried out on fresh haemolysates of the propositi and their relatives. The enzyme activity of the haemolysates was measured according to the above-mentioned report [16]. The electrophoresis of G-6-PD was carried out on strips of cellulose acetate gel according to the technique described by RATTAZZI *et al.* [14].

All the female relatives of the propositi were also tested for the cellular localization of G-6-PD activity by the methaemoglobin elution technique [6], as the heterozygotes for G-6-PD deficiency are often indistinguishable from normal females by their enzyme activity assayed on haemolysates.

Studies of G-6-PD variants. All the variants, defined by high decrease of enzyme activity and normal electrophoretic mobility were considered as belonging to the Mediterranean group because in the region of Ferrara, G-6-PD deficiency is commonly due to this type of variant [12].

On the contrary further investigations were carried out in two deficient hemizygotes which had the same type of G-6-PD variant electrophoretically faster than normal. Fresh blood samples of 80–100 ml collected in ACD were drawn from the propositi. The enzyme was characterized according to the methods recommended by the WHO Standardization Committee [16]. The samples were partially purified by chromatography on diethylaminoethyl cellulose. The enzyme preparations were stored at +4 °C as precipitates in ammonium sulphate 43.0% (w/v) and dialysed against the appropriate buffers when required. Michaelis constant (K_m) for G-6-P and NADP [2], relative rate of utilization of the substrate analogues (2-deoxy-G-6-P and deamino-NADP) [3], and pH dependence of G-6-PD activity in the range 5.5–10.5 [4], were determined.

Comparative electrophoretic mobility on starch gel of normal G-6-PD B and of the variant samples were carried out on three buffer systems: phosphate, pH 7.0 [1]; Tris-HCl, pH 8.8 [13], and Tris-EDTA-boric acid, pH 8.6 [16]. Heat stability was determined after 20 min incubation at 47 °C in presence of NADP and bovine albumin at final concentration of 1 mg/ml.

Results

Of 2,500 males screened, 2,460 (98.40%) had normal erythrocyte G-6-PD activity. 38 males (1.52%) had severe G-6-PD deficiency due to variants electrophoretically indistinguishable from normal, and considered of the Mediterranean type. Two males (0.08%) had severe enzyme

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Glucose-6-Phosphate Dehydrogenase Ferrara

A New Variant of G-6-PD Identified in Northern Italy

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Key Words. Erythrocyte enzymes · G-6-PD deficiency · G-6-PD Ferrara · Malaria · Neonatal jaundice

Abstract. A new variant of glucose-6-phosphate dehydrogenase (G-6-PD) has been discovered in Northern Italy, in the district of Ferrara. This variant is characterized by high decrease of red blood cell enzyme activity (less than 5% of normal), high affinity for G-6-P and NADP, increased utilization of deamino-NADP and 2-deoxy-G-6-P and faster electrophoretic mobility in the buffer systems commonly used for the classification of the G-6-PD variants. The new G-6-PD type was never associated with clinical manifestations in any cases except neonatal jaundice in some of the newborns with this enzyme deficiency. The frequency of the new variant in the Ferrara district indicates that it has probably appeared in this area by mutation some centuries ago. It is suggested that this variant should be named G-6-PD Ferrara.

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is present at endemic levels in Northern Italy near the delta of the Po river, district of Ferrara. This area was marshy and malarial for 8 centuries, starting from the great flood of the Po river in 1150 AD until the drainage carried out at the beginning of this century [7]. The selective pressure of malaria acting here for at least 30 generations, made the G-6-PD deficiency of the Mediterranean type endemic in this area [12] and may also favour any other variant which probably appeared in the country by mutation or by immigration. In fact, besides the common Mediterranean variant, several local types of G-6-PD have been recently described in different areas of the Mediterranean basin where G-6-PD deficiency is endemic [9, 15, 17]. The identification of new variants, increasing the knowledge about the characteristics of different G-6-PD alleles, is mainly of importance in order to understand the variety of clinical manifestations due to this enzyme defect in terms of molecular pathology [10].

Table I Characterization of G-6-PD Ferrara

Enzyme type	Red cell enzyme activity % of normal	Electrophoretic mobility % of normal			K_m G-6-P μM	K NADP μM	2d-G-6-P utilization % of G-6-P rate	Deamino-NADP utilization % of NADP rate	Heat stability	pH optima
		TEB	Tris-HCl	phosphate						
Normal B	100	100	100	100	50–70	2.9–4.4	<4	50–60	normal	normal
Ferrara	<5	105	106	117	28–29	2.8–3.9	12 23	65–86	very low	peak t 9 0

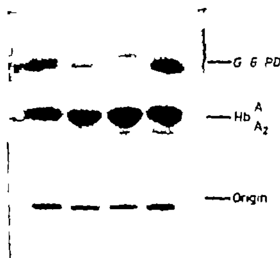


Fig 1 Electrophoretic patterns of G-6-PD B and Ferrara on cellulose acetate. *From left normal homozygous female (Gd^B/Gd^B) heterozygous female ($Gd^B/Gd^{Ferrara}$)* deficient hemizygous male ($Gd^{Ferrara}$) normal male (Gd^B). The shadowed bands just before dark bands of HbA in the two normal samples are due to 6-phosphogluconate dehydrogenase.

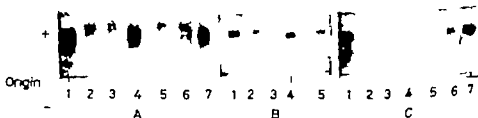


Fig 2 Comparative electrophoreti patterns of purified G-6-PD B and G-6-PD Ferrara on starch gel. A – Phosphate buffer pH 7 0 (2.5 V/cm, 18 h at +4 °C). B – TEB buffer (Tris-EDTA-borate), pH 8.6 (3.0 V/cm, 18 h at +4 °C) C – Tris HCl buffer pH 8.8 (2.5 V/cm 18 h at +4 C) Samples 1 4 and 7 – G-6-PD B, 2, 3 5 and 6 – G-6-PD Ferrara.

deficiency due to a variant electrophoretically faster than normal This variant was also present in some of their relatives, and was the same for the two family groups although they shared no common ancestor back for 6–8 generations, as resulting from the registers of births and marriages.

As malaria was endemic in this country during the last 8 centuries and may have favoured this variant with selective advantage equal to 0 1 ac

naemia. The lack of acute haemolytic incidents following the ingestion of certain drugs or vegetables could be accidental, as the small number of subjects with G-6-PD Ferrara since identified may never have met or ingested substances possibly haemolytic for this type of variant.

On the contrary the absence of chronic haemolytic anaemia, which requires no exogenous cause, is not accidental, and may be explained by the high affinity of G-6-PD Ferrara for its substrate and coenzyme. In fact, several G-6-PD variants are asymptomatic in spite of their very low enzyme activity provided they have high affinity for G-6-P. As recently pointed out by YOSHIDA [18] this kinetic property plays an important role at the low substrate concentrations within the erythrocyte, while the *in vitro* activity is less important as it is measured with optimal substrate concentrations.

On this basis, G-6-PD variants with severe enzyme deficiency can be divided in two groups [17, 18]: the first group of variants which have unusually low affinity for the natural substrate and permit severe red cell damage, and the second one of variants with no chronic haemolytic manifestations and high affinity for G-6-P and NADP. Since G-6-PD Ferrara is precisely characterized by high affinity for its substrate and coenzyme and by absence of chronic haemolytic anaemia, although its *in vitro* activity is less than 5% of normal, it would be included in the second group together with the variants Mediterranean, Union, Markham, and others.

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cording to the model proposed by CAVALLI SFORZA and BODMER [5], the frequency of this allele was expected to be about 10^{-8} 8 centuries ago. Such a frequency corresponds to that of new alleles due to the mutational activity of the G-6-PD structural locus [5] suggesting that this variant may be a local one first appearing in this region by mutation.

In fact the characterization of the new G-6-PD type has demonstrated that it is different from any other variants identified up till now [17] and, therefore it has been named G-6-PD Ferrara. This variant shows the following characteristics (table I) (a) red blood cell G-6-PD activity lower than 5% of normal (b) K_m for physiological substrate and coenzyme lower than normal (c) increased utilization of the alternative substrates (2-deoxy-G-6-P and deamino-NADP) (d) normal (truncate) pH optimum curve with a peak at 9 0 (e) electrophoretic mobility increased in all buffer systems used, and (f) marked heat instability with no activity detectable after 20 min incubation at 47 C.

Among the relatives of the propositi 5 other hemizygous males and 8 heterozygous females had the same type of variant. The presence of the variant was revealed by laboratory procedures only as no clinical manifestations accompanied the enzyme deficiency in adult persons. In fact no cases of drug-induced anaemia or mild chronic haemolytic anaemia [8] were present in any family group Only some cases of hyperbilirubinaemia in the first week of life for subjects with G-6-PD Ferrara would be related with this enzyme defect, as no other causes of neonatal jaundice had been ascertained [11]

Discussion

A large mass screening carried out in the district of Ferrara Northern Italy has revealed a new G-6-PD variant different from any other variants previously described. Since this variant has not been revealed by population surveys performed in other areas of the Mediterranean basin where G-6-PD deficiency is endemic [9 15 17] it is most likely to be considered a local variant. This is supported by the fact that new G-6-PD deficient alleles due to mutation and favoured by malaria [5] could reach the frequency we have found for this variant just in the period during which malaria was endemic at Ferrara.

The new variant, which would be named G-6-PD Ferrara, is characterized by a high decrease of enzyme activity but accompanied by no haemolytic phenomenon except for rare cases of neonatal hyperbilirubi-

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Cytoplasmic Fibrils in Plasma Cell Leukemia

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Key Words. Cytoplasmic fibril · Myeloma · Plasma cell leukemia · Pleural effusion · Ultrastructure

Abstract. In a patient with plasma cell leukemia, associated with pleural effusion, ultrastructural studies of the peripheral blood plasma cells showed an abundance of cytoplasmic fibrils. The nature of fibrils was not clarified, but they were not amyloid fibrils. This finding, coupled with a literature review, suggests that the cytoplasmic fibrils in plasma cells may be an additional cytological feature of this rare form of leukemia.

Plasma cell leukemia is a rare variant of multiple myeloma characterized by large numbers of circulating plasma cells in the blood. We recently observed a case of plasma cell leukemia associated with pleural effusion. Ultrastructural studies of the circulating plasma cells of the patient revealed an abundance of cytoplasmic fibrils.

Case Report

The patient, a 59-year-old man, was referred to the Veterans Administration Hospital, Charleston, S.C., on June 14, 1974, with complaints of fatigue, dyspnea, and multiple petechiae of 4 weeks duration. The pulse was 104/min, and the temperature was 100.3 °F. Multiple petechiae and signs of left pleural effusion were noted: hemoglobin 15.2 g/100 ml, WBC 18,000/µl, platelet count 100,000/µl. About 50% of the blood leukocytes were immature plasma cells containing nucleoli. Fluid obtained by thoracentesis was serosanguineous and contained plasma cells. Electrophoresis of the serum and the pleural fluid revealed the presence of a monoclonal protein in the gamma region, identified as IgA, type L. The serum level of IgG was 240 mg/100 ml, IgA 2,530 mg/100 ml, and IgM 5 mg/100 ml. The pleural fluid content of IgG was 140 mg/100 ml, IgA 2,080 mg/100 ml, and IgM 1 mg/100 ml. Bence-Jones protein was not present in the urine; blood urea nitrogen 28 mg/100 ml, blood

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Cytoplasmic Fibrils in Plasma Cell Leukemia

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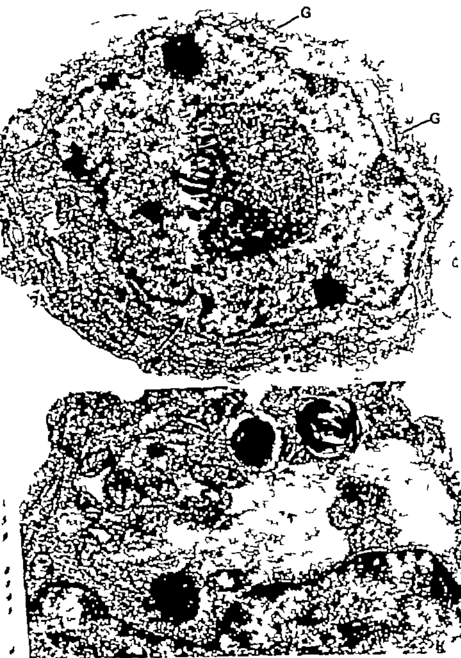
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glucose 184 mg/100 ml serum calcium 12.5 mg/100 ml, and albumin 2.500 mg/100 ml, uric acid 13.2 mg/100 ml. Skeletal X ray survey disclosed many radiolucent areas in the skull. Immature plasma cells almost totally replaced normal cell types in an aspirate of iliac bone marrow. Pleural biopsy revealed pleural thickening with fibrosis and sheet like aggregates of similar plasma cells. The patient was placed on oral medication consisting of melphalan 6 mg daily prednisone 60 mg daily allopurinol 300 mg daily and isoniazid 300 mg daily. The hypercalcemia was treated with oral phosphate solution, furosemide, and intravenous infusion with normal saline. Although the hypercalcemia and hyperuricemia improved, the patient continued to accumulate fluid, and died on July 4 1974.

At autopsy leukemic infiltration by plasma cells was seen in a number of organs including liver lungs, thyroid, and adrenals but not in the kidneys. Tissues from the liver kidneys, tongue, and the rectal mucosa were negative for amyloid when examined under a polarizing microscope with Congo-red staining.

Materials and Methods

Buffy coat cells were separated from the heparinized blood by light centrifugation and were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 C for 90 min. The cells were then rinsed with the buffer and fixed with cacodylate-buffered 2% osmium tetroxide for 90 min. They were dehydrated through graded alcohols and propylene oxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate lead citrate sequence and examined in an Hitachi HS-8 electron microscope.

Epon sections were stained with alkaline toluidine blue and with Congo-red for examination by a polarizing microscope as described by SHIRAHAMA and COHEN [19].

Results

The profile of a typical plasma cell in the circulation is shown in figure 1. The immaturity of the nucleus is illustrated by its large size, sparse heterochromatin and the presence of nucleoli. The majority of the cells displayed prominent cytoplasmic fibrils which were most commonly located in the perinuclear region and often occupied extensive cytoplasmic areas

Fig. 1. The plasma cell profile encloses a large nucleus with sparse heterochromatin and prominent nucleolus (Nu). The relatively less abundant cytoplasm (consistent with an increased nuclear/cytoplasmic ratio) contains cisternae of granular reticulum in parallel array several widely separated small Golgi complexes (G) and bundles of fibrils (arrow). × 14,600.

Fig. 2. An extensive area in this plasma cell consists of closely packed fibrils. Additional features include dense bodies with myelin figures and mitochondria with dense matrix particles. × 22,300.


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ma cells in our patient afforded no evidence for the fibrils consisting of amyloid. In addition, the ultrastructural features of the fibrils were not entirely consistent with those reported for amyloid fibrils [20] Moreover the patient exhibited no clinical evidence of amyloidosis and such deposits were not found at autopsy Although bundles of fibrils have been reported in the cytoplasm of normal plasma cells, they were usually small and infrequent [12] Cytoplasmic fibrils have also been observed in marrow plasma cells of a few patients with myeloma [13 21, 22] but not in those from a large number of patients with multiple myeloma [2, 4 5 7 10, 22, 25] The incidence of these fibrils in plasma cell leukemia, however appears to be extremely high. Prominent cytoplasmic fibrils have been reported in the circulating plasma cells from 4 [1 3, 8, 24] out of 6 [1, 3 8 18, 23 24] cases with plasma cell leukemia examined ultrastructurally The presence of such fibrils may be related to the leukemic presentation of this disorder

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(fig. 1 2) The characteristically abundant cisternae of rough-surfaced endoplasmic reticulum were generally arranged in parallel array (fig. 1). The mitochondria contained one to four small dense granules (fig. 2) and multiple Golgi areas were noted within a single cell (fig. 1)

The light microscopic stains failed to provide evidence for amyloid in the plasma cells, since they neither stained with toluidine blue nor showed characteristic green birefringence under a polarizing microscope when stained with Congo-red.

Discussion

Plasma cell leukemia is reported to occur in 1–2% of the cases of multiple myeloma [9 14] Although such a leukemia is extremely rare, its unique clinical features have been firmly established. Patients resist to chemotherapy and have a poor prognosis [14] The aggressive nature of this neoplastic process is suggested by the high labeling index of plasma cells when studied with tritiated thymidine [17] and in this case was further substantiated by the plasma cell infiltration of the pleura and the monoclonal protein in the pleural fluid

Pleural effusion as a complication of multiple myeloma is extremely rare. Recently SAFA and VAN ORDSTRAND [16] reported a myeloma patient who like our patient, presented with pleural effusion Their case also had plasma cell infiltration of the pleura and monoclonal protein in the pleural fluid. These authors found only five cases of myeloma associated with pleural involvement. The incidence of pleural effusion in plasma cell leukemia is not known PRUZANSKI *et al* [14] reported the presence of pleural effusion in 1 of 10 observed cases, but others [6 9 11 15] did not report such complications in their literature review Pleural effusion appears to be a rare complication of plasma cell leukemia.

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Cytochemical Studies on T and B Lymphocytes and Lymphoblasts with Special Reference to Acid Phosphatase

Helmut Wehinger and Walter Möbius

Universitätskinderklinik and Medizinische Universitätsklinik, Freiburg i.Br

Key Words Acid phosphatase Acute lymphatic leukemia B cells β-Glucuronidase Cytochemistry Leukemia Rosette-forming lymphocytes T cells

Abstract. Lymphoblasts from ten patients with ALL or stage IV malignant lymphoma were studied cytochemically and investigated for rosette-forming capacity with sheep red blood cells (SRBC) as T cell surface marker. SRBC binding capacity and acid phosphatase or β-glucuronidase were tested simultaneously in single lymphocytes isolated from normal blood donors. Our results suggest that the presence of acid phosphatase (and β-glucuronidase) represents functional state of lymphocytes or lymphoblasts qualitatively independent of T cell differentiation, but quantitatively more pronounced in T cells than in B lymphocytes or non-T lymphoblasts.

The capacity of leukemic lymphoblasts to form rosettes with sheep red blood cells (SRBC) has been accepted to differentiate T cell leukemia or T cell malignant lymphoma from non-T variants of lymphoblastic leukemia (ALL) About 20°/ of pediatric ALL patients have T cell ALL [2–6, 9 17 18] Older age, a preference for male sex, a mediastinal mass, and a very high initial leukocyte count, are the clinical features typical for T cell ALL in comparison to non-T ALL [18].

A similar pattern of clinical manifestations was found for a type of childhood ALL characterized cytochemically by a strong acid phosphatase reaction, the so-called acid phosphatase type of ALL [1 7 12] Recent evidence suggests that the acid phosphatase type of ALL might be

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phoblasts. 29,900 WBC with 62% lymphoblasts were found in the peripheral blood. Studies on *normal lymphocytes* were performed on cells from five healthy blood donors.

Methods

Isolation of lymphocytes 3 ml of heparinized blood + 3 ml of PBS, 3 ml of cerebrospinal fluid (CSF), or diluted bone marrow suspension in PBS with 100 U/ml heparin were layered over 2 ml Ficoll-metrizoat (24 parts 9% Ficoll + 10 parts 34% Rompacon 75%), and centrifuged at room temperature for 40 min at 400 g. Only round bottomed tubes were used. After centrifugation, the lymphocyte band was pipetted off, resuspended in PBS, and washed three times. Then the suspension was adjusted to 5×10^6 cells/ml. The recovery rate was 55 ± 14%. The contamination by monocytes, basophils, etc., was 19 ± 12%.

Fresh SRBC were stored in Alsever' solution at 4 °C for maximum of 14 days. A 1 % suspension in PBS was prepared before use.

Human AB-serum was heated to 56 °C for 30 min, incubated with 1% SRBC at 37 °C for 15 min and for a further 60 min at 4 °C. After centrifugation, the supernatant was stored in small amounts at 20 °C.

Rosette test. Lymphocytes (5×10^6/ml), SRBC (1%), and serum were preincubated at 37 °C for 5 min. 0.1 ml SRBC and 0.1 ml lymphocytes were added to 0.02 ml of serum. The mixture was kept at 37 °C for 5 min, centrifuged at 400 g for 5 min, and left in refrigerator at 4 °C for 18 h. The sediment was then cautiously brought into suspension, put under a coverslip and examined for rosette-forming cells. Disregarding monocytes and granulocytes, 200 lymphocytes were counted. The viability of cells judged by trypan blue exclusion was 98–100%.

T obtain slide preparations, 0.02 ml of the suspension with rosetted cells were added to 4.8 ml PBS with 10% fetal calf serum. 0.2 ml of this diluted suspension were filled into the well of cytocentrifuge and spun at 700 rpm for 5 min. The slides were then stained with May-Grünwald-Giemsa or cytochemically. When comparing the rosette counts from either suspension or slide preparation, agreement was only obtained for the percentage of lymphocytes with zero or three and mor attached SRBC. No correlation between the two methods was found when the percentages of lymphocytes binding 1 or 2 SRBC were compared. Apparently accidental neighborhood of 1 or 2 SRBC to one lymphocyt could not be distinguished from true attachment. Therefore, in suspensions as well as on slides, only lymphocytes with three or more SRBC were considered T lymphocytes and counted as such. The normal range in peripheral blood was 67 ± 8% (mean ± SD).

Cytochemistry I addition to May-Grünwald-Giemsa stains, reactions for acid phosphatase [8] and β-glucuronidase [13] were performed on the slide preparations of rosetted cells. Single cells could now be evaluated for the capacity to bind SRBC, and for cytochemical parameters, simultaneously. Only cells with granular reaction products were counted as positive. The strength of the reaction within positive cells was not taken into consideration. PAS stains gave unreliabl results. Glycogen is apparently broken down during isolation procedures. This process may be prevented by the addition of NaF but at the cost of high rate of cell deaths.

Table I Clinical data of our ten patients at the time of diagnosis and duration of their first complete remission (CR) from day of diagnosis

Patient No	Sex/Age years	WBC/μl	Spleen cm	Lymph node enlargement	Mediastinal mass	CR months
1	F/6	1 100	6	+	0	8+
2	M/7	1 700	0	0	0	4
3	M/7	13 600	2	+	0	8
4	M/12	275,000	6	+	0	10
5	F/11	4 900	0	0	+ +	25
6	M/6	2,300	0	+	0	50
7	M/1	44,000	5	+	0	8
8	M/9	10 600	1	+	0	23
9	M/2	330 000	8	+	0	7+
10	M/10	29 900	0	+	(+)	3

identical with T cell ALL [4 5 15 16] If a strong acid phosphatase reaction should prove to be a reliable T cell marker the distinction between the two types of ALL would become easier because instead of fresh cell suspensions only dried bone marrow smears would be needed.

We examined the capacity of lymphoblasts from patients with ALL or malignant lymphoma to bind SRBC as a T cell marker as well as their cytochemical profile Normal lymphocytes were tested for rosette-forming capacity and acid phosphatase or β-glucuronidase by methods allowing for evaluating these parameters simultaneously in single cells. Our results caution against taking acid phosphatase as a T cell marker at the present stage of knowledge.

Patients

Ten patients aged 1 12 years, nine with ALL and one patient (No. 10) with leukemic transformation of a hitherto untreated lymphosarcoma were studied (for clinical data see table I). Three patients were investigated with initial manifestation of their disease, and seven patients with leukemic relapse. In these seven patients, cytochemical data had been obtained previously but were not complete. Patient No. 10 presented with a left-sided pleural effusion on Feb 26, 75 No mediastinal mass was seen on the chest X ray The bone marrow was free of blast cells. On April 17 75, a biopsy of mediastinal lymph nodes was taken, and the diagnosis of a malignant lymphoma was made The bone marrow was now infiltrated with 70% atypical lym-

Table III Sequential cytochemical profile in four patients with non-T-ALL, range of acid phosphatase positivity 0–89%

Patient No.	Date of diagnosis	Date of cytochemistry	Source of blast cells	Percent blast cells positive for acid phosphatase	β-glucuronidase	PAS
4	11-20-73	11-20-73	BM	ND	<1	16
		2-6-75	CSF	<1	6	20
		2-18-75	BM	<1	<1	33
5	7-9-73	7-9-73	BM	0	59	12
		7-29-75	BM	14	2	24
6	12-27-68	2-28-73	BM	2	73	100
		3-18-75	BM	26	72	100
		3-18-75	CSF	65	91	100
		8-12-75	BM	0	94	100
7	1-25-72	9-12-72	CSF	0	0	80
		2-15-74	CSF	ND	0	78
		5-2-75	BM	89	49	68
		8-8-75	BM	34	100	36

Table IV Acid phosphatase and β-glucuronidase in T and B lymphocytes of healthy blood donors

No. of SRBC close to one lymphocyte	Lymphocyte subpopulation	No. of lymphocytes evaluated	No. of acid phosphatase positive cells (%)
Acid phosphatase			
0	B	234	121 (52)
1	B+T	141	82 (58)
2	B+T	101	60 (59)
3+	T	1,268	1,056 (83)
β-Glucuronidase			
0	B	224	139 (62)
1	B+T	134	100 (75)
2	B+T	96	83 (87)
3+	T	756	714 (94)

α-Naphtylesterase showed weak positivity with no difference between T and non-T lymphoblasts.

Normal lymphocytes. Normal lymphocytes were evaluated for the number of adherent SRBC and the presence or absence of acid phosphatase or β-glucuronidase simultaneously (fig. 1, table IV). No significant

Table II T cell markers and cytochemistry in leukemic blast cells from bone marrow (BM), peripheral blood (PB) or CSF

Patient No	Source of blast cells[1]	Percent of blast cells positive for			
		T cell markers	acid phosphatase	β-glucuronidase	PAS
1	BM a	0	0	0	0
	BM b	1	0	55	0
3	PB b	2	1	0	22
4	CSF b	0	0	6	20
5	BM b	1	14	2	24
6	BM b	0	26	72	100
7	BM b	0	23	100	36
8	CSF b	0	74	100	0
9	PB a	77	94	28	1
10	PB a	65	96	76	14

[1] a = Initial manifestation b = relapse after therapy

Results

Lymphoblasts The blast cells from bone marrow peripheral blood, or CSF in eight patients did not bind SRBC, and in two patients they did (table II) In four patients, rosette tests were repeated on different occasions or with blast cells from different sources The results were identical The two patients with positive rosette tests were considered to have T cell malignancy The WBC was high but no massive mediastinal mass was present.

Both these patients had an over 90% positive acid phosphatase reaction. Acid phosphatase was present both in lymphoblasts with and in those without attached SRBC in cytocentrifuge preparations.

Acid phosphatase was also found in half of the non T cases, with counts between 14 and 74% at the time of the rosette test (table II) Acid phosphatase reactions were repeatedly performed in four of the non-T ALL cases on different occasions during the course of their disease or on blast cells from different sources Varying results ranging from 0 to 89% positive blast cells were obtained (table III) T lymphoblasts in part were positive for β glucuronidase (table II) The PAS score was low In non T lymphoblasts the range of β-glucuronidase and PAS-positivity was 0–100% (table II III) Peroxidase stains were negative in all instances.

higher percentage in T cells. Our results suggest that the presence of acid phosphatase represents a functional state of lymphocytes or lymphoblasts qualitatively independent of T cell differentiation, but quantitatively more pronounced in T cells than in B lymphocytes or non-T lymphoblasts. HIRSCHHORN *et al.* [10] have shown that acid phosphatase-rich granules may be induced by PHA in human lymphocytes.

Thus, rather than the presence *per se* it is the higher percentage of positive cells which might discriminate between T and non-T malignancies. This argument has been expressed in a previous report [5]. The critical level is probably 90%, a higher percentage being found in T ALL and a lower percentage in non-T ALL. CATOVSKY [4] suggests to consider ALL cases with 75–100% acid phosphatase-positive blast cells as of T cell origin. He never found more than 30% acid phosphatase-positive blast cells in non-T ALL. These results, however, are at variance with ours, possibly because our study was not restricted to initial bone marrow findings. One of CATOVSKY's patients [5] is of special interest in this context. This patient's blast cells were stimulated by PHA and 99% were positive for acid phosphatase. Though SRBC receptors were absent, the patient was classified as T ALL. This classification is equivocal, since PHA may stimulate B cells as well as T cells [14]. No T ALL with negative acid phosphatase has been reported so far.

The unspecificity of the presence of acid phosphatase is further stressed by the fact that nonlymphoid cells, i.e. myeloid precursors, monocytes, and erythroblasts contain acid phosphatase [11]. In fact, a strong acid phosphatase reaction has been claimed to be characteristic of undifferentiated acute erythremic myelosis [17].

Further work on larger series of patients is needed to determine the degree of correlation between strong acid phosphatase reactions and the presence of T cell surface markers. At the present time, the rosette test with SRBC cannot be replaced by the acid phosphatase reaction in order to differentiate between T and non-T lymphatic malignancies.

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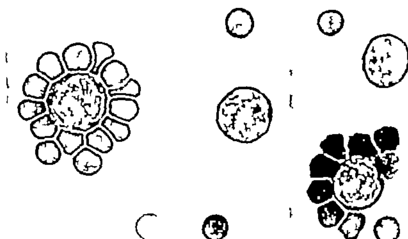


Fig. 1 Acid phosphatase stain performed on normal lymphocytes after rosetting. Positive T cell (left), positive B cell (middle), positive B cell and negative T cell (right)

differences were noted between single blood donors. Therefore cumulative figures are given in table IV Both enzymes were present in B cells as well as in T cells, though in a higher percentage in T cells.

Discussion

Among 10 patients with ALL or stage IV malignant lymphoma, we found two with T cell surface markers on their blast cells. Peripheral blast cell counts were high and more than 90% of the blast cells gave a strongly positive acid phosphatase reaction. These findings confirm the reports in the literature on T cell ALL [5 16 18] A mediastinal mass – lacking in our patients – is not an obligatory sign of T cell ALL. Acid phosphatase was not only positive in T cell ALL. Up to 89% of lymphoblasts in 4 of 8 non T ALL cases gave positive acid phosphatase reactions. The percentage of positive blast cells varied from patient to patient, during the course of the disease in single patients, and between blast cells from bone marrow peripheral blood, or cerebrospinal fluid.

Normal lymphocytes – whether belonging to the B or T subpopulation – both contained acid phosphatase (and β-glucuronidase) though in a

higher percentage in T cells. Our results suggest that the presence of acid phosphatase represents a functional state of lymphocytes or lymphoblasts qualitatively independent of T cell differentiation, but quantitatively more pronounced in T cells than in B lymphocytes or non-T lymphoblasts. HIRSCHHORN *et al.* [10] have shown that acid phosphatase-rich granules may be induced by PHA in human lymphocytes.

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Further work on larger series of patients is needed to determine the degree of correlation between strong acid phosphatase reactions and the presence of T cell surface markers. At the present time, the rosette test with SRBC cannot be replaced by the acid phosphatase reaction in order to differentiate between T and non T lymphatic malignancies.

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Meningeal Relapse after Long-Term Remission in Acute Childhood Lymphocytic Leukemia

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Key Words. Childhood leukemia · Leukemia therapy · Lymphoblastic leukemia · Meningeal leukemia

Abstract. Central nervous system (CNS) relapse is reported in three children with acute lymphocytic leukemia who received intermittent prophylactic CNS therapy with intrathecal methotrexate. The children were on monochemotherapy either with methotrexate or 6-mercaptopurine for 2 / years. The CNS relapse occurred 2 /, 10 and 11 months after cessation of all chemotherapy. Irradiation and/or intensive chemotherapy including drugs as BCNU and Ara-C which are known to cross the blood-brain barrier were not given. Preventive CNS radiotherapy should be considered in all children who did not receive an adequate prophylactic CNS therapy even after long-term remission before chemotherapy is stopped.

With prolongation of survival of childhood lymphocytic leukemia, involvement of the central nervous system (CNS) is observed more frequently [6]. Patients whose complete remission is terminated by a CNS relapse have less chance for a cure of their disease [5, 13]. With prophylactic cranial irradiation and intrathecal methotrexate injections, a 15-fold reduction of the incidence of CNS leukemia has been achieved [3]. Craniospinal irradiation in doses of 2,400 rad gave equally good results [4]. Periodic administration of intrathecal methotrexate with no radiotherapy and intensive systemic chemotherapy has recently been shown to be as effective as irradiation to prevent CNS leukemia [8]. However, despite prophylactic CNS therapy with intrathecal methotrexate the occurrence of meningeal leukemia in three children will be reported in this paper.

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Asymptomatic CNS relapse occurred 10 months later. Lumbar puncture showed 7 cells/μl (16% blasts).

Discussion

CNS leukemia, especially meningeal involvement, is the most frequent site of initial relapse in childhood lymphocytic leukemia and may later give rise to hematologic relapse by repopulation of lymphoblasts from the CNS in the marrow. More recently its diagnosis is made upon routine lumbar punctures often before any clinical symptoms appear. With improved laboratory diagnosis lymphoblasts are identified in the spinal fluid often without the presence of pleocytosis [1, 7, 10]. CNS leukemia was detected in 2 of the 3 reported patients by a routine lumbar puncture, the third patient had signs of increased intracranial pressure. Cerebrospinal fluid cell count was only minimally increased in the two girls and was normal in the boy. Lymphoblasts were seen on Giemsa stained cytocentrifuged liquor cerebrospinalis.

In an extensive histopathologic study, the earliest evidence of CNS leukemia was shown to be localized in the superficial arachnoid and the perivascular arachnoid which extends deeply within the brain. With more advanced arachnoid leukemia, the deep arachnoid is infiltrated, and invasion into brain tissue may occur through destruction of the pia-glial membrane [12]. To eradicate all leukemic cells in the arachnoid prophylactic, CNS radiotherapy has been integrated in the treatment plan of childhood acute lymphocytic leukemia [9]. Therapy with 2,400 rad cranial irradiation and intrathecal methotrexate (12 $mg/m^2 \times 5$ doses) given early in remission drastically reduced the incidence of CNS relapse. Of 31 children receiving this mode of therapy only three relapsed in the CNS [2]. Smaller doses of cranial irradiation did not alter the rate of subsequent CNS disease [9]. Cranial irradiation plus irradiation of the spinal axis (2,400 rad) had the same effect as cranial irradiation and intrathecal methotrexate but was associated with more hematologic toxicity [4]. No adverse effect on intellectual performance, psychological and neurological functions were seen with irradiation of the CNS [14]. Intrathecal methotrexate coupled with an extensive systemic chemotherapy including drugs like BCNU and Ara-C which are known to cross the blood-brain barrier can also effectively prevent the development of CNS leukemia. From 70 patients treated with a multiple drug chemotherapy regimen three had a relapse of the disease in the CNS [8].

Case Reports

Case 1

A 2 1/2 year-old girl was admitted with 1 month history of fatigue, anorexia and anemia. Physical examination revealed pallor, ecchymoses of the lower extremities and moderate cervical and inguinal lymphadenopathy. Hemoglobin was 7.3 g%, WBC 8,400/μl with 46% blasts and thrombocytes 21,000/μl. In the marrow aspirate 93% lymphoblasts were counted. Induction of remission therapy with weekly injections of vincristine 2 mg/m² i.v. and prednisone 40 mg/m² daily per os for a period of 5 weeks was given. As consolidation therapy *l*-asparaginase 1,000 U/kg i.v. × 10 days followed the vincristine-prednisone treatment and the patient was subsequently in remission. Maintenance therapy consisted of methotrexate 25 mg/m² *per os* twice a week for 1 year and 6-mercaptopurine 2.5 mg/kg daily for 18 months. Prophylactic CNS therapy was started with intrathecal methotrexate 12 mg/m² every 6 weeks after 6 months in remission. Reinduction with vincristine and prednisone was given once a year. All chemotherapy including intrathecal methotrexate injections was stopped after 30 months of continuous complete remission. 1/2 months later CNS relapse with 27 cells/μl (85% blasts) in the liquor cerebrospinalis occurred.

Case 2

A 5-year-old girl had parotitis and was admitted with the tentative diagnosis of acute lymphoblastic leukemia. She complained of fatigue, poor appetite and abdominal pains. Physical examination revealed a severely ill anemic child with generalized lymphadenopathy, hepatomegaly (6 cm) and splenomegaly (8 cm). Hemoglobin was 9.7 g%, WBC 6,500/μl with 83% lymphocytes and no blasts, thrombocytes 190,000/μl. Marrow aspirate showed 23% lymphoblasts. After a 5-week induction therapy with vincristine 2 mg/m² i.v. once a week and prednisone 40 mg/m² *per os* daily, the child went in remission and maintenance therapy with methotrexate 25 mg/m² *per os* twice a week was initiated. In addition, a reinduction therapy with vincristine and prednisone once a year and prophylactic CNS therapy with intrathecal methotrexate 12 mg/m² every 6 weeks were given for a period of 30 months. 2 1/2 years after achieving remission, systemic chemotherapy was stopped and 11 months later meningeal relapse with headache and vomiting was detected. Lumbar puncture revealed 500 cells/μl with 50% blasts.

Case 3

A 5-year-old boy was admitted with a history of fever, anorexia, pallor and pains in the legs. Physical examination revealed generalized purpura, lymphadenopathy, hepatomegaly of 6 cm and splenomegaly of 3 cm. Hemoglobin was 5.2 g%, WBC 7,200/μl (45.5% blasts), thrombocytes 230 000/μl. Marrow aspirate revealed 91% lymphoblasts and in the liquor cerebrospinalis no cells were found. The patient received vincristine 2 mg/m² i.v. once a week for 5 weeks and prednisone 40 mg/m² *per os* daily for 5 weeks. Following remission methotrexate 25 mg/m² *per os* twice a week was given and reinduction therapy with *vincristine* and prednisone was added once a year. After 1 year therapy intrathecal methotrexate 12 mg/m² every 6 weeks was started. A total of six doses were injected. All chemotherapy was stopped after 1/2 years of continuous complete remission.

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The prevention of CNS leukemia significantly increased the long-term leukemia free remission in children with acute lymphoblastic leukemia [5] After cessation of chemotherapy children who did not receive prophylactic cranial irradiation relapsed more frequently than children receiving prophylactic CNS radiotherapy [5] It is not yet clear if factors such as age at diagnosis and initial WBC have a predictive value for the occurrence of relapse [5 11] A controlled cooperative study which was recently started may answer this question

The three children reported above had only intermittent intrathecal methotrexate injections for prophylaxis of CNS involvement. They were treated with methotrexate or 6-mercaptopurine as monotherapy and did not receive CNS irradiation The likelihood of developing CNS relapse appeared to be small considering the age, the initial WBC, and the lack of mediastinal mass at the time of their diagnosis. It is questioned if CNS leukemia could have been prevented and complete remission prolonged in these three children, if CNS irradiation had been given before cessation of systemic chemotherapy

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ly cirrhosis [3]. In addition, the enlargement of the spleen is partially the result of the increased red cell destruction. In children with homozygous β-thalassemia, splenectomy has been advocated as a form of symptomatic therapy [4]. The indications for the removal of the spleen in thalassemia are still not sufficiently clarified, particularly regarding the short- and long-term effects on the degree of iron deposition in the liver and in other organs, with the possible harmful consequences. The present study was undertaken to evaluate the extent and the interrelationship of iron deposition in the liver and the spleen in homozygous β-thalassemia, and the effect of splenectomy on iron deposition in the liver.

Materials and Methods

Patients. The patients represent a homogeneous group, being descendents of parents born in the same area of Kurdistan and living in Jerusalem. All were under the same medical care from birth or early childhood. There were two pairs of siblings: cases No. 1 and 3, 9 and 10.

Diagnostic criteria. The diagnosis of β-thalassemia major was made on the basis of typical facial malformations, impaired growth and development, typical morphological changes of peripheral red blood cells and increase in fetal hemoglobin and/or hemoglobin A_2 [4].

Availability of tissue specimens of livers and spleens. Liver tissue for histological examination was obtained during splenectomy (4 cases), with a needle shortly after death (1 case) or at autopsy (3 cases). Splenic tissue was obtained by means of splenectomy (10 cases) or at autopsy (1 case). One patient underwent splenectomy elsewhere 7 years before postmortem examination and the specimen was not available.

Histological examination. Tissues from the livers and spleens were processed according to routine procedures. Multiple sections cut at 5 μm were stained with hematoxylin and eosin, as well as for reticulum and for hemosiderin. The amount of hemosiderin in the liver and the spleen was classified into four subgroups according to the intensity of the specific iron stain, which was evaluated in histological sections [7]. All the sections were evaluated independently by two of the authors.

Results

Clinical and Laboratory Data

Eight of the children were males and four were females. Their ages at time of splenectomy or at the time of death were between 2 and 14 years. Each patient required blood transfusions shortly after birth. Their hemoglobin level between transfusions was in the range of 7–9 g%. The total

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Splenectomy, Iron Overload and Liver Cirrhosis in β-Thalassemia Major

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Key Words. Hemosiderosis Liver cirrhosis Splenectomy Thalassemia

Abstract In order to evaluate the interrelations of splenectomy iron overload and cirrhosis, histologic specimens of liver and spleen were examined and correlated in 12 children with β thalassemia major All patients had received blood transfusions since infancy Correlations seemed to exist between splenic hemosiderosis and splenic weight, and between the latter and the age at time of splenectomy All liver samples showed varying hemosiderosis, not correlated with the number of transfusions or the children s age. Irregular liver cirrhosis existed in three children, 7 8, and 14 years after splenectomy No cirrhosis existed in any of the children where the spleen was *in situ* Splenectomy in children with thalassemia may carry the long-term risk of liver cirrhosis.

One of the major complications in homozygous β-thalassemia results from increased iron deposition in numerous organs, particularly in the reticuloendothelial system (RES) This is due, at least in part, to ineffective erythropoiesis and severe constant hemolysis from early infancy The severe anemia in patients with the homozygous form of the disease requires frequent blood transfusions which contribute an additional amount of iron to parenchymal tissues of various organs and to the RES [1] The liver and spleen are the major components of the RES and therefore accumulation of iron is most prominent in these organs.

The iron overload from both external and internal sources, as well as the extramedullary hematopoiesis which is progressive with the years, are the main causes for the marked enlargement of both liver and spleen in homozygous β-thalassemia [2] In the liver increased iron deposition results in hemosiderosis, with gradual development of fibrosis and eventual

Table I Summary of pertinent clinical and histological findings in liver and spleen in 12 children with β-thalassemia major

Case No.	Sex	Age 1 1975 or at death, years	Age at time of splenectomy years	Total No. of transfusions (packed RBC)	Spleen weight g	Spleen hemosiderosis	Spleen Gamna-Gandy bodies	Spleen extramedullary hematopoiesis	Liver hemosiderosis general	Liver hemosiderosis parenchymal	Liver hemosiderosis Kupffer cells	Liver hemosiderosis portal space	Liver hemosiderosis periphery of lobules vs center	Liver extramedullary hematopoiesis
1	F	4½	4	35	220	1+		1+	2+	2+	1+		1+	1+
2	M	6	4½	45	950	4+	2+	2+						
3	M	6	5½	45	550	3+	2+	2+	1+	1+	3+	2+		2+
4	F	8	6	33	240	2+	2+	1+	4+	4+	3+	3+	3+	–
5	M	10	2	70	320	1+		3+	4+	4+	3+	3+	cirrhosis	2+
6	M	10	7	47	850	4+	4+	3+						
7	M	10	7½	31	750	3+	1+	2+						
8	M	11	9	50	200	2+	1+	1+						
9	M	13	11	60	1 050	3+	2+	3+	2+	2+	2+	2+	2+	2+
10[a]	F	14		80	1 660	4+	2+	3+	2+	2+	2+	–	1+	2+
11	F	16	2	150	490	1+		3+	3+	3+	3+	2+	cirrhosis	1+
12	M	21	14	180	specimen not available				4+	4+	4+	4+	cirrhosis	2+

Until splenectomy or death.

Degrees of excess iron in periphery of lobules compared with centrilobular siderosis.

Died complete postmortem examination was done.

Died multiple samples were obtained from several organs.

numbers of blood transfusion until the time of splenectomy are listed in table I Hemoglobin analysis and other pertinent data have been reported elsewhere [5 6] There was no clinical evidence of hepatitis in any of the children under study

Correlation between Histological Findings in the Spleen Age at Time of Splenectomy Transfusion Requirements and Splenic Weight

All spleens were enlarged with a weight ranging between 200 and 1 660 g Seven spleens weighed more than 400 g. There was a direct correlation between the degree of splenic hemosiderosis and the weight of the spleens There was some relation between the age of the children at the time of splenectomy or autopsy (case 10), and the splenic weight. the average age of the seven children with a spleen weighing more than 400 g was 7 3 years and that of the four children with smaller spleens was 5.2 years However some of the younger patients had very large spleens (e.g., patients No 2 and 3) There was no correlation between the total number of transfusions and the degree of hemosiderosis or the spleen weight.

Extramedullary hematopoiesis was found in all spleens. Its degree could not be directly correlated with the spleen weight, although in the three smallest spleens (200 220 and 240 g) extramedullary hematopoiesis was less prominent than in the larger ones. Siderotic nodules, also known as Gamna-Gandi bodies, were found in 8 of 11 spleens examined. There was no correlation between their number and the other parameters.

Correlation between Histological Findings in the Liver Age at Time of Examination and Transfusion Requirements

There was a variable degree of hemosiderosis in the eight livers that were examined without direct correlation between the age, the total number of transfusions, and the degree of hemosiderosis found both in parenchymal cells and in the RES It was not possible to correlate the degree of hemosiderosis in the latter two cell types (table I) There was no correlation between the degree of over-all hemosiderosis in the liver and in the spleen (table I) However a similar extent of hemosiderosis was found in the RES of the liver and the spleen.

The distribution of iron in the center or the periphery of the liver lobules was related directly to the total amount of iron in the parenchymal cells In the cases with a marked degree of hemosiderosis, more hemosiderin granules were found in the peripheral hepatocytes than in the center of the lobule (fig. 1) Hemosiderin granules were also found in macro-

Table 1 Summary of pertinent clinical and histological findings in liver and spleen in 12 children with β-thalassemia major

Case No.	Sex	Age in 1975 or at death, years	Age at time of splenectomy years	Total No. of transfusions (packed RBC)[1]	Spleen weight g	Spleen hemosiderosis	Spleen Gamna-Gandy bodies	Spleen extramedullary hematopoiesis	Liver hemosiderosis general	Liver hemosiderosis parenchymal	Liver hemosiderosis Kupffer cells	Liver hemosiderosis portal space	Liver hemosiderosis periphery of lobules vs center[2]	Liver extramedullary hematopoiesis
1	F	4½	4	35	220	1+		1+	2+	2+	1+		1+	1+
2	M	6	4½	45	950	4+	2+	2+						
3	M	6	5½	45	550	3+	2+	2+	1+	1+	3+	2+		2+
4	F	8	6	33	240	2+	2+	1+	4+	4+	3+	3+	3+	
5	M	10	2	70	320	1+		3+	4+	4+	3+	3+	cirrhosis	2+
6	M	10	7	47	850	4+	4+	3+						
7	M	10	7½	31	750	3+	1+	2+						
8	M	11	9	30	200	+	1+	1+						
9	M	13	11	60	1 050	3+	2+	3+	2+	2+	2+	2+	2+	2+
10[3]	F	14		80	1 660	4+	2+	3+	2+	2+	2+	–	1+	2+
11[3]	F	16	2	150	450	1+		3+	3+	3+	3+	2+	cirrhosis	1+
12[4]	M	21	14	180	specimen not available				4+	4+	4+	4+	cirrhosis	2+

[1] Until splenectomy or death.
[2] Degrees of excess iron in periphery of lobules compared with centrilobular siderosis.
[3] Died; complete postmortem examination was done.
[4] Died; multiple samples were obtained from several organs.



phages within the portal spaces. Approximately the same degree of extramedullary hematopoiesis was found in 4 of the 5 children where the liver and spleen were examined simultaneously

In three patients (cases 5 11, 12) who are no longer living, the liver was examined 8, 14 and 7 years after splenectomy respectively These three cases exhibited a similar and striking finding at autopsy: marked hemosiderosis both of the hepatocytes and the liver RES, and, in addition, typical macronodular irregular cirrhosis In each case, the normal liver architecture was completely replaced by small and large regenerative nodules separated by wide bands of fibrous tissue (fig. 2). In none of these children who were controlled regularly during their whole life, was clinical evidence for hepatitis ever found. No cirrhosis was found in children of the same age group who had not been splenectomized at the time of examination of the liver (cases 9 10).

Discussion

Increased iron overload in homozygous β-thalassemia is becoming a major limiting factor of life expectancy since blood transfusions are given more frequently to maintain approximately normal hemoglobin levels [1 4] It is generally accepted [1] that frequent blood transfusions have not only a favorable influence on the growth and development of thalassemic patients, but also prolong their life. Therefore, the need to minimize or prevent iron accumulation and its deleterious effects on different organs is now generally acknowledged as a major problem in the management of thalassemic patients [8]

Among the major organs which are affected by the iron overload are the liver and spleen. Therefore, in children who are more frequently transfused and who are reaching adolescence, one would expect higher levels of iron in these organs. This was not evident from the present series where, in a few cases from a rather homogenous group, there was a high

Fig. 1 Extensive siderosis of liver with fine granules of hemosiderin in hepatocytes and coarse granules in macrophages in portal spaces (case 4) The concentration of hemosiderin granules is higher in the hepatocytes in the periphery of the liver lobule than in the more centrally localized hepatocytes. Gomori' iron reaction $\times$ 250.

Fig 2 Liver with irregular macronodular cirrhosis in case 5 The liver was examined 8 years after splenectomy Laidlaw reticulum stain. $\times$ 42.

degree of hemosiderosis in the liver and/or spleen in spite of a moderate transfusion requirement and a relatively young age (cases 2, 4). Moreover in three other children with the same transfusion requirement, the degree of hemosiderosis was considerably less (cases 1 3 9). BARRY *et al* [7] on the other hand found a direct correlation between the transfusion requirement and an increased deposition of iron in the liver. In order to explain this difference in results, one must bear in mind that storage iron is not only derived from blood transfusions but also from ineffective erythropoiesis and constant hemolysis. It is also possible that the different methodology employed in the present and the other study [7] is the reason for the different results. Although in the present study the degree of hemosiderosis was not measured quantitatively the same results were obtained during examination of old sections, and when new sections were cut from all blocks, stained simultaneously for iron and examined independently by two observers.

The distribution of iron between the liver and the spleen is of major significance in thalassemia Most of the iron derived from transfused red blood cells is stored in the cells of the RES in the liver and spleen. From the RES the iron is redistributed via the serum iron compartment, mainly to the hepatocytes [8 9] It seems feasible that after splenectomy more iron derived from transfused red blood cells will be stored in the Kupffer cells in the liver These cells will therefore, become more rapidly overloaded, so that any additional iron will be added to the serum iron compartment, and larger amounts of iron will now be deposited in the hepatocytes, leading to liver cell damage and eventually to cirrhosis [8]

In children where liver tissue was examined at the time of splenectomy there was usually only little or moderate hemosiderosis of hepatocytes (cases 1 3 9] This was also a prominent finding at the autopsy of case 10 a 14 year-old girl with an intact spleen The one exception was case 4 where the hepatocytes contained larger amounts of iron at the time of splenectomy In contrast, there was severe hemosiderosis in the hepatocytes in livers examined 8 14 and 7 years after splenectomy (cases 5 11 12)

The significance of the large quantities of iron in the liver cells lies in its causative role in the possible development of liver fibrosis and cirrhosis. It was shown that patients treated with iron-chelating agents with a consequent decrease of liver hemosiderosis had less liver fibrosis than an untreated group [7] Considering the fact that several groups have men

tioned an increase in iron deposition in the liver following splenectomy in thalassemia [3 10] and that the degree of iron overload is an important causative factor in the development of cirrhosis [8] it seems logical to expect a higher incidence of cirrhosis in splenectomized patients. In the present study cirrhosis appeared only in the three patients where splenectomy was performed 7 8, or 14 years before examination of the liver No cirrhosis was found in nonsplenectomized patients, even in those where the liver was examined at the age of 11 and 14 years (cases 9 10). The same apparent relationship between splenectomy and the development of liver cirrhosis was mentioned in one case from the series of BHAMARAPRAVATI *et al.* [11] and, in an additional case described by BERRY and MARSHALL [10] However these authors did not place any special emphasis on the implications of this finding. WITZLEBEN and WYATT [3] found a tenfold increase in the hepatic iron content in one child with thalassemia during a period of 6 months after splenectomy and they suggested that this operation may lead to development of cirrhosis.

The risk of possible development of cirrhosis in children with thalassemia several years after splenectomy must be taken into account when this mode of symptomatic therapy is considered, and weighed against its positive effects [6]

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degree of hemosiderosis in the liver and/or spleen in spite of a moderate transfusion requirement and a relatively young age (cases 2, 4). Moreover in three other children with the same transfusion requirement, the degree of hemosiderosis was considerably less (cases 1 3 9). Barry *et al* [7] on the other hand, found a direct correlation between the transfusion requirement and an increased deposition of iron in the liver. In order to explain this difference in results, one must bear in mind that storage iron is not only derived from blood transfusions but also from ineffective erythropoiesis and constant hemolysis. It is also possible that the different methodology employed in the present and the other study [7] is the reason for the different results. Although in the present study the degree of hemosiderosis was not measured quantitatively the same results were obtained during examination of old sections, and when new sections were cut from all blocks, stained simultaneously for iron, and examined independently by two observers.

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The Effect of Ionophore on Platelet Aggregation in von Willebrand's Disease and in Congenital Afibrinogenemia. A Comparison with Ristocetin

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Key Words. Afibrinogenemia · Blood coagulation · Ionophores · Platelet aggregation · Ristocetin · Von Willebrand's disease

Abstract. Platelet aggregation in citrated and heparinized plasma by Ionophore A 23187 and Ristocetin was studied in normal subjects and in patients with von Willebrand's disease and congenital afibrinogenemia. Aggregation by Ionophore was normal in all groups both in citrated and heparinized plasma. Aggregation by Ristocetin in citrated plasma was normal in congenital afibrinogenemia, in normal subjects and in types II and III of von Willebrand's disease. It was absent in classical von Willebrand's disease, type I. In heparinized plasma it was absent in all groups, except in some patients with von Willebrand's disease, type III. It is concluded that Ionophore A 23187 behaves in a different way than Ristocetin and has no diagnostic implications.

Von Willebrand's disease has drawn considerable attention in recent years. Several variants and subtypes have been demonstrated, but a satisfactory classification of the condition has not been achieved yet. Ristocetin has been recently proposed as a useful diagnostic tool in von Willebrand's disease [11]. Using Ristocetin and citrated or heparinized blood we have been able to classify von Willebrand's patients in three classes [9]. Some patients (type I) do not show any platelet aggregation in citrated and heparinized blood. A second group (type II) is characterized by a normal aggregation pattern in citrated plasma and no aggregation in heparinized plasma. This pattern is also seen in normal subjects. Finally, a third group (type III) is characterized by a normal aggregation in both anticoagulants. The significance of such classification remains to be fully

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Table I Main features of three patients with congenital afibrinogenemia

Patients	Fibrinogen levels mg %	Fibrinogen levels, antigen	Factor VIII AHF % of normal	Factor VIII AGN, % of normal	Factor VIII VWF U/100 ml	Platelet adhesiveness to glass, %	Bleeding time min	Platelet aggregation by Ristocetin (1.5 mg/ml), % ctr 1:10	Platelet aggregation by Ristocetin (1.5 mg/ml), % hep. 50 IU
Normal values	250–500	70–130	60–160	60–160	65–100	20–50	2 5	74 (±12.5)	0 or near 0
R. P	unassayable	0	90	70	95	8	12	71	0
M. M.	unassayable	0	100	100		7	7	70	0
A. S.	unassayable	0	110	140			14		

Table I Main factors of six patients with von Willebrand's disease

Patients	Factor VIII AHF % of normal	Factor VIII AGN % of normal	Factor VIII VWF U/100 ml	Platelet adhesiveness to glass, %	Bleeding time min	Platelet aggregation Ristocetin (1.5 mg/ml), citr 1:10	hep 5
Normal values	60–160	60–160	65–100	20–50	2–5	74 (±12.5)	0 or [illegible]
B. L.	20	10	11	6	7	absent	0
B. V.	14	10	7.5	11	11	absent	0
G. C.	14	10	10	3	9	absent	0
G. F.	1[illegible]	12.5	1.5	5	10	78	0
C. A.	15	34	33	3	> 20	79	7[illegible]
C. I.	16	45	41	0	> 20	69	60

evaluated but it emphasizes further the impression that von Willebrand's disease is a very complex condition [10, 12].

Congenital afibrinogenemia is a rare coagulation disorder. Platelet aggregation in this condition has been reported as variably defective. In our three patients with this disorder we have found that platelet aggregation to adrenaline in citrated plasma was defective, whereas that to Thrombofax was absent. Aggregation to ADP and collagen was only slightly defective whereas aggregation to Ristocetin was normal. In heparinized blood an improvement of the aggregating pattern was noted but the adrenaline-induced aggregation remained defective.

The studies on von Willebrand's disease and congenital afibrinogenemia indicate that both factor VIII antigen and fibrinogen are important cofactors in platelet aggregation to one or more than one aggregating agents [1, 4, 7, 11–13, 17, 19, 21]. Ionophores have been recently proposed as platelet-aggregating agents. These compounds seem to transfer calcium ions through cell membranes and to free them from intracellular deposits. Since it was shown that ionophores act on the muscular sarcoplasmatic reticulum, the possibility of a similar action on the platelet-contractile system has been formulated [16, 22]. Ionophores could free calcium ions from intracellular deposits, making them available for platelet contraction and subsequent release and aggregation. It seemed interesting to study whether this compound, which is an antibiotic as Ristocetin, could add some information to the study and classification of von Willebrand's disease and congenital afibrinogenemia.

Table I. Main features of three patients with congenital afibrinogenemia

Patients	Fibrinogen levels mg %	Fibrinogen levels, antigen	Factor VIII AHF % of normal	Factor VIII AGN, % of normal	Factor VIII VWF U/100 ml	Platelet adhesiveness to glass, %	Bleeding time min	Platelet aggregation by Ristocetin (1.5 mg/ml), % cttr 1 10	Platelet aggregation by Ristocetin (1.5 mg/ml), % hep. 50 IU
Normal values	250–500	70–130	60–160	60–160	65–100	20–50	2 5	74 (±12.5)	0 or near 0
B. P	unassayable	0	90	70	95	8	12	71	0
M. M	unassayable	0	100	100		7	7	70	0
A. S	unassayable	0	110	140			14		

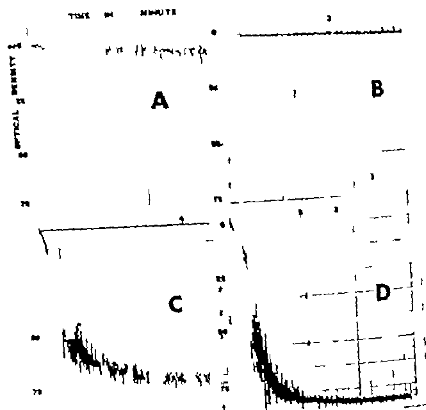


Fig. 1. Platelet aggregation by Ristocetin (1.5 mg/ml) and by Ionophore (0.4 M) in a patient with von Willebrand's disease, type I. *A* No aggregation by Ristocetin in citrated plasma. *B* No aggregation by Ristocetin in heparinized plasma. *C* Aggregation by ionophore in citrated plasma. *D* Aggregation by ionophore in heparinized plasma, similar to C.

Material and Methods

Material and methods have been discussed in detail elsewhere [7, 8]. Only new data will be given herein.

Ionophore A 23187 was kindly supplied by Dr. BOTTECCHIA, Institute of Physiology, Padua, Italy, and by Dr. R. L. HAMILL, Eli Lilly Laboratories, Indianapolis, Ind. The ionophore was dissolved in ethanol and added to the platelet samples (final concentration of ethanol 0.1 / v/v) at a final concentration of 0.4 M.

Assay of von Willebrand factor VIII was evaluated by a modification of the method proposed by WEISS *et al.* [20]. Washed platelets were obtained by the method of WALSH [18] with albumin density gradient separation. The separation was repeated four times. The test system consisted of 0.8 ml of final washed platelet sus-

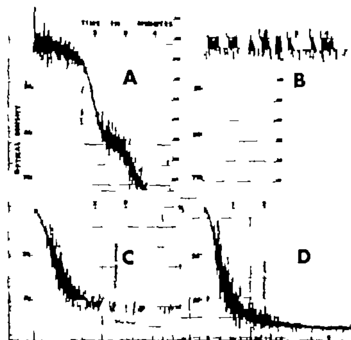


Fig. 2. Platelet aggregation by Ristocetin (1.5 mg/ml) and by ionophore (0.4 μM) in patient with von Willebrand' disease, type II. *A* Aggregation by Ristocetin in citrated plasma. *B* No aggregation by Ristocetin in heparinized plasma. The same pattern is seen in heparinized plasma of normal subjects. *C* Aggregation by ionophore in citrated plasma. *D* Aggregation by ionophore in heparinized plasma.

pension (about 300,000/ l) plus 0.2 ml of normal or test plasma plus 0.1 ml of Ristocetin solution (1.5 mg/ml).

The platelet-induced aggregation by ionophore was carried out in citrated (1 10 dilutions of whol blood) and heparinized plasma (50 IU/ml of whol blood [8]).

The following patients were studied. 10 normal subjects of both sexes, 6 patients with von Willebrand's disease, and 3 patients with congenital afibrinogenemia. The main data of the patients are summarized in tables I and II.

Results

Platelet aggregation to ionophore was normal in all von Willebrand's disease patients. The aggregation curves obtained were similar to their

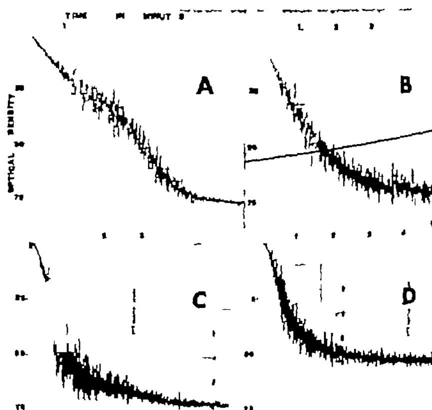


Fig. 3. Platelet aggregation by Ristocetin (1.5 mg/ml) and by ionophore (0.4 μM) in a patient with von Willebrand's disease type III. *A* Aggregation by Ristocetin in citrated plasma. *B* Aggregation by Ristocetin in heparinized plasma. *C* Normal aggregation by ionophore in citrated plasma. *D* Normal aggregation by ionophore in heparinized plasma.

normal counterparts and were different from patterns obtained with Ristocetin (fig. 1–3). The latter aggregating agent produced no aggregation in classical von Willebrand's disease (type I) (fig. 1) regardless of the anticoagulant used. In von Willebrand's disease type II Ristocetin gave a normal aggregation in citrated plasma and no aggregation in heparinized plasma (fig. 2). In von Willebrand's disease type III Ristocetin gave a normal aggregation with both anticoagulants (fig. 3).

In congenital afibrinogenemia, similarly normal results were obtained. The curves were similar to those noted with Ristocetin and higher than the curves obtained with adrenaline, collagen and Thrombofax (fig. 4).

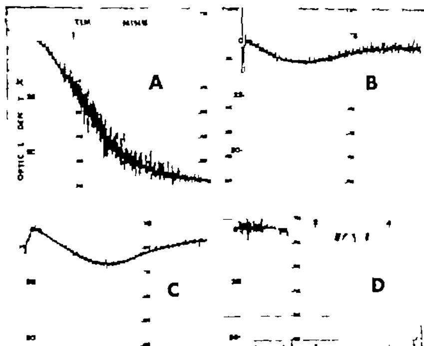


Fig 4 Platelet aggregation in patient with congenital afibrinogenemia. *A* Aggregation by ionophore (0.4 μM) in citrated plasma. *B* Aggregation by adrenaline (65 μg/ml) is slightly defective and no second wave is visible. *C* Aggregation by ADP (0.5 μg/ml) is also slightly defective and no second wave is visible. *D* Aggregation by Thrombofax is very decreased or absent. Platelet aggregations to collagen and to Ristocetin are not reported. They were only slightly defective or normal, respectively

Discussion

Our results indicate that ionophore acts in a different way as compared to Ristocetin, in von Willebrand's disease patients. A normal aggregating pattern was obtained in every instance. The different behavior from Ristocetin appears *striking. Our von Willebrand's patients had* variable levels of factor VIII antigen, a variable behavior to Ristocetin but a uniform normal aggregating pattern with regard to ionophore.

Our normal results in congenital afibrinogenemia patients indicate that fibrinogen is not needed for ionophore aggregation. Our patients had a

complete fibrinogen deficiency [7] and therefore it may be assumed that, had even traces of fibrinogen been needed, platelet aggregation would be defective Therefore, in this condition, the pattern is similar to that observed with Ristocetin

The mechanism of action of ionophore is not completely clarified as yet. Recently it was supposed that the aggregating activity of one of these compounds (x 537A) may be independent from calcium ions [22] This remains to be proven and seems unlikely in view of our results. Other authors [5 21] have, in fact, found no difference in the mechanisms of action of x 537A and of A 23187 the compound used by us. On the contrary at least two sites, one plasmatic and one on platelet membrane, have been proposed for Ristocetin [3] Our studies indicate that the ionophore-induced platelet aggregation does not depend on factor VIII antigen or part thereof and from fibrinogen and has no similarity to Ristocetin induced platelet aggregation. Our negative results are in agreement with the observation that washed normal platelets show a normal aggregation by ionophore.

Unfortunately ionophore studies have not allowed any further information about the two diseases studied Preliminary results show also that platelet aggregation by ionophore is normal in hemophilia A and in combined factor V and VIII deficiency It may be concluded that ionophores are not useful as a diagnostic tool

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Studies on Human Platelets Stored at 20–22 C without Agitation

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Key Words Platelet functions Platelet storage Platelet transfusion Thrombocytes

Abstract Human blood platelets in ACD plasma were stored in sterile plastic bags for 24–96 h at the ambient temperature without agitation. No spontaneous aggregation nor bacterial contamination were noted A progressive loss of the following parameters was seen, platelet count ADP thrombin collagen-, and epinephrine-induced platelet aggregation platelet factor 3 activity· reversible response to the osmotic shock volumetric constants amount of UV absorbant material ^{14}C 5-hydroxytryptamine and ^{3}H-adenosine uptake and release· platelet population pattern and glycogen synthesis activity The platelet aggregation and release, the osmotic shock test, and the platelet population pattern appear to better illustrate the early changes during platelet storage and to account for the 25–42% of recirculation of 24 h stored platelets administered into thrombocytopenic patients. As stated by Murphy and Gaardner, platelets stored at 20–22 °C with or without agitation, although having failed to retain total functional and biochemical capacities, paradoxically seem to recuperate *in vivo* as shown by survival data and hemostatic effects.

There is increasing evidence of a better preserved platelet morphology [1–3 6, 11 19 20] physiology and survival time [2, 9 12, 17] at ambient [11 19–21 27 28] rather than at low temperature. The *in vivo* and *in vitro* experimental data reported by Murphy and Gaardner [19] and others [11 27] have focused attention on the advantage of storing platelets at room temperature. These authors proposed storage with gentle stirring under controlled condition But there is now sufficient documentation showing that stirring activates platelets and thus causes significant morphophysiological changes of cells [18 23]

These assumptions justified us in undergoing an experimental work on platelet storage at ambient temperature without agitation. We report in this paper the results concerning some physical biochemical and transfu-

sional properties of human platelets stored at 20–22 C without agitation. It appears that these platelets presently allow to respond to the increasing clinical demands for platelet transfusion despite the partial loss of their biochemical and functional properties.

Material and Methods

Normal human venous blood was collected in plastic bags (Medicoplast, France), containing 70 ml of ACD solution, A formula (NIH, Bethesda, Md.).

Platelet-rich plasma (PRP) was prepared by centrifuging the total blood at 80–100 *g* at 10 °C for 20 min in a KP63 Jouan centrifuge. The supernatant was expressed in second bag, containing 70 ml ACD-A solution (final pH 6.5) and then stored without stirring at the ambient temperature for 24–96 h [28]. Platelet-poor plasma (PPP) was obtained by the centrifugation of aliquots of PRP at 20,000 *g* at 5 °C for 30 min in an RC2B Sorvall refrigerated centrifuge. Platelet counts were performed by phase contrast microscopy [5] after the PRP dilution in the Unopette device (Becton, Dickinson). Platelet suspension is contaminated by approximately 30 red blood cells and 3 white blood cells per 10^4 platelets.

Platelet aggregation was measured by the turbidimetric method of BORN and CROSS [4] and O'BRIEN [22] in an EEL 401 Evans photometer connected to Vitatron UR 400 recorder for the aggregation tracings. The stock solutions were prepared in $HNaCO_3$-buffered saline, pH 7.3±0.1 as follows: $2 \times 10^{[illegible]}$ M^{-1} ADP $1 \times 10^{[illegible]}$ M^{-1} epinephrine in 0.1 N HCl, 1 U ml^{-1} thrombin, collagen gel in $1.67 \times 10^{[illegible]}$ M^{-1} acetic acid (according to HOLMSEN *et al.* [13]), and aliquots were stored at –20 °C until use.

The *osmotic shock test* [8] was carried out by using platelets in plasma according to the procedure previously described [10, 15], based on the response of platelets to the reduction of the osmotic pressure of the plasmatic milieu (from 300 to 200 mosm). Figure 1 represents the mode of calculation of the A, C, R parameters and their significance, allowing to quantitate the reversibility of the process.

Volume distribution of fresh and stored platelets have been determined by using a Coulter counter Z Bic coupled with C 1000 Analyzer [24] (K_J = 0.336). The platelet volume distribution in the range of 1.7–33.3 µm was analyzed from the unregistered curves. The relative percentage in respects to the 'mode' the curve mode value, and the platelet mean volume were then calculated.

Platelet factor 3 (PF3) free and total activities were determined by the clotting time measurement after recalcification, as described by RABINER and HRODEK [25], before and after three times repeated freezing (at 70 °C) and thawings (at 37 °C) of PRP [7]. The appearance of gelatinous mass was taken as the reaction end point. In each experiment standard curves were prepared, using dilutions of citrated human PPP pooled from 4 to 5 healthy individuals and stored at 70 °C.

The amount of platelet total *UV-absorbent material* was measured in the perchloric acid extracts obtained by treating the PRP and PPP with a 12% (v/v)

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Studies on Human Platelets Stored at 20–22 °C without Agitation

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These assumptions justified us in undergoing an experimental work on platelet storage at ambient temperature without agitation. We report in this paper the results concerning some physical, biochemical and transfu

method of LOWRY [16]. In all cases the data obtained at day 0 of platelet storage were taken as 100%, and the results obtained during the platelet storage were expressed as percentages from this maximum.

Chemicals. Adenosine diphosphate Na-salt (ADP), *l*-epinephrine, collagen from bovine achilles tendon, serotonin-creatinine sulfate, adenosine, sucrose, Tris (hydroxy) methylaminomethane, uridine-diphosphoglucose (UDPG), G6P glycyl-glycine, glycogen from shellfish type I, albumine bovine F V were from Sigma Chemicals, St. Louis, Mo. Bovine thrombin 50 IU mg⁻¹ was from Hoffman-La Roche, Basel. Packard Soluen TR 100 and NEN Aquasol were used. ^{14}C-5HT 58 mCi/mM, and ^{3}H-adenosine, 50 mCi/mM, were from CEA and UDP U ^{14}C-glucose was purchased from the Radiochemical Center Amersham, England. All other chemicals were of reagent grade.

Results

Platelet counting data. These show a gradual but moderate decrease of the platelet count during storage at 22 C without stirring. The decrease varied from 2–3 / after 24 h to 10–12% after 96 h storage in respect to the platelet count at day 0. No spontaneous aggregation nor bacterial contamination were noted (300 examinations have been performed on various batches of stored platelets).

Platelet aggregation. Table I summarizes the results obtained when the aggregation was measured in the presence of various aggregating agents during the platelet storage. In all cases a gradual loss of the platelet-induced aggregation was noted during the 24–96 h of storage. It can be seen that after 24 h of storage the platelet aggregation, induced by higher concentration of ADP (5×10^{-6} M and 10^{-5} M), is still 92 and 80% from the aggregation of the fresh platelets (day 0 of storage). The aggregation velocity decreases to 36 and 30% after 48–96 h of storage. The rate and extent of the reversible aggregation, induced by lower concentrations of ADP (5×10^{-7} M and 10^{-6} M), were significantly reduced at 24 h of storage. When the platelets initially present a double wave of aggregation in the presence of 10^{-6} M ADP this ability is retained only up to 24 h of storage. The data presented (table I) also show that the platelet aggregation induced by collagen, epinephrine and thrombin, was more than 50% reduced after 24 h of storage and was completely abolished after 96 h a practically similar platelet behaviour has been noted by using these three agents at the indicated concentration.

Osmotic shock. Figure 1 shows a typical light transmission pattern obtained when a platelet suspension is submitted to the osmotic shock,

refrigerated $HClO_4$ solution. After the centrifugation of the mixtures for 20 min at 20 000 *g* at 5 °C, the clear supernatants were used for the light absorbance measurement at 260 nm in a Zeiss PMQ2 spectrophotometer. A standard curve was prepared, using various concentrations of adenosine (stock solution 10^{-3} M^{-1}) in plasma treated like the test assays.

Measurement of ^{14}C 5-hydroxytryptamine (5HT) and ^{3}H-adenosine uptake by platelets in plasma (2.5–3 × 10^{8} platelets ml^{-1}) was carried out at 37 °C by using ^{14}C-5HT (58 $\mu Ci/\mu M$, CEA, France, 1 10^{-6} M^{-1} 5HT) [28]. At 0- and 30-min intervals after the addition of the labeled compound, the reaction was stopped by adding 10 mM serotonin in 0.1 ml buffered saline, containing 0.4% EDTA, and immediately centrifuged in an Eppendorf centrifuge for 1 min at 12,000 *g*. The platelet buttons were washed twice with EDTA–5HT saline solution and finally solubilized in 0.5 ml Soluen TM100 (Packard). The radioactivity of the solubilized platelets as well as that of 0.1 ml of the supernatant, obtained from the first centrifugation, was determined by liquid scintillation counting in a Packard Tricarb spectrometer in the presence of 10 ml of Aquasol (NEN). The adenosine uptake by platelets was determined by the platelet incubation with 8-^{3}H-adenosine (50 mCi mM^{-1}, CEA, France, 0.1 μCi 5 μM^{-1}) for 10 min at 37 °C in a 1 ml final volume. The reaction was stopped by adding 10 mM 'cold' adenosine in buffered saline. All other conditions were like those used in the ^{14}C-5HT uptake experiments.

Measurement of ^{14}C-5HT and ^{3}H-adenosine release by platelets in plasma was done by incubation with labeled compounds for 30 min at 37 °C as previously described. Radioactive material that had not been taken up by the platelets was separated by centrifugation (1 min at 12,000 *g* in an Eppendorf centrifuge). Platelet pellets were washed twice with buffered saline solution by centrifugation and resuspended in 1 ml of Tris-buffered saline, pH 7.4. 1-ml aliquots of the labeled platelet suspensions were then incubated at 37 °C with magnetic stirring in the presence of 0.1 ml collagen solution (50 μg). After 0 and 5 min of incubation the platelet suspensions were refrigerated for 5 min at 0 °C, centrifuged for 1 min at 12,000 *g* and the radioactivity determined on 0.1-ml aliquots of the supernatant. The results ($cpm^{-1}/10^{8}$ platelets) were calculated in respect to the total radioactivity of platelets (control) and expressed as percentages of the maximum (the release of labeled serotonin or of total labeled nucleotides at day 0 of storage).

Platelet population separation was carried out as described [6] by using the ultracentrifugation of platelets in plasma on sucrose density gradients. The protein content of the four individual platelet fractions A, B, C, D isolated following their increasing densities was checked by light absorbance at 280 nm in a Zeiss PMQ2 spectrophotometer after dilution in Tris-buffered saline. The results (arbitrary units of $OD_{280\ nm}$) were calculated as percentages in each fraction from the total.

Measurement of glycogen synthesis. The two variants of platelet transglucosylase EC 2.4.1.11 D (b) dependent from glucose-6-phosphate (G6P) and I' (an independent on G6P) were determined in the four platelet populations as previously described [29]. The glycogen content was estimated according to the method of SEIFTER *et al.* [29] by using a glucose solution as the standard. The transglucosylase specific activity (counts, mg protein $^{-1}$ min^{-1}) was expressed as percentages of the total in the four fractions.

The *protein content* was measured in aliquots of washed platelet fractions by the

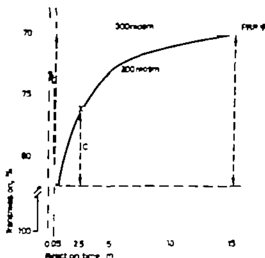


Fig 1 A typical pattern of the light transmission through platelet suspension in plasma as function of time, after the induction of the osmotic shock. Experimental conditions: to 1 vol of platelets in plasma (3–4 × 10⁸ platelets ml⁻¹) 2 vol of distilled water ere added (or physiological saline for the control). The modification of the light transmission was measured at 610 nm (Jouan Spectral Junior type L spectrophotometer). The results were expressed in arbitrary units (%) of the light transmission. A = Light transmission at 0.5 min· C = light transmission at 2 min 5 sec; R = light transmission at 15 min after the osmotic shock induction. The parameters A, C, R are then calculated [15].

PF3 activity In our experimental conditions, the mean values for PF3-free and total activity were 58.6 ± 4 and 36.8 ± 5 sec, respectively No significant changes of these activities occurred in the PRP for 24–96 h as compared to the basal values at day 0 time of storage. In all cases, both free and total PF3 activities were lower in the ACD-PRP than in the citrated PRP (results not shown).

The platelet total UV-absorbant material estimated by the indicated procedure does not present a significant modification during storage (results *not presented*).

Uptake and release of 5HT In the experimental conditions described 80–85% of the labeled serotonin is taken up by the fresh platelets. The corresponding values were arbitrarily taken as maximum activity (100%). The platelet stored for 24 h at 20 C present a slightly elevated or non-

Table I Effect of storage at 20–22 °C without agitation on the platelet aggregation induced by various agents

Duration of storage h	Aggregation inducer ADP M^{-1} 5×10^{4}	10^{4}	5×10^{6}	10^{6}	collagen 20 µg ml^{-1}	epinephrine 5 µg ml^{-1}	thrombin 0.1 U ml^{-1}
0	100	100	100	100	100	100	100
24	92	80	74	44	45	46	46
48	82	74	56	39	30	24	29
72	61	51	36	19	16	5	6
96	36	30	15	8	0	0	0

The aggregation mixture contained 0.9 ml PRP ($2.5–3.10^{7}$ platelets ml^{-1}) and 0.1 ml of aggregating agent at the indicated concentration. All experiments were carried out at 37 °C with magnetic stirring and results were calculated as percent velocity [4]. The recovered activity during platelet storage was then expressed as percentages from the control (day 0 of storage) and represent mean values of five experiments.

caused by the reduction of the osmotic pressure of the plasmatic milieu. The initial increase of the light transmission at 30 sec (A parameter) is followed by a decrease at 2 min 30 sec (C parameter) that stabilizes at 15 min after the water addition (R parameter) At day 0 of storage the C/A ratio representing the degree of reversibility of the platelet response to the osmotic shock, is 0.52 ± 0.05 ($p < 0.001$) A decrease of 30% of this parameter was obtained at 24 h of storage. This decrease of the ability of stored platelets to reversibly respond to the osmotic shock becomes 50 and 60% after 48 and 72 h of storage respectively The R/A ratio, which represents the reversibility of the process at 15 min after the osmotic shock induction decreased linearly with the duration of platelet storage, like the C/A ratio (fig. 2)

Platelet volume repartition. The volume repartition of platelets stored at more than 24 h (48 and 72 h) presents a family of typical volume distribution curves which show a slight shifting to the right, to the large elements as compared to the volume distribution curve of fresh platelets. Table II shows that the modes did not vary during 48 h and increased from 4.3 ± 0.2 to 4.6 ± 0.3 after 72 h storage. The mean platelet volume varied slightly during the first 48 h (from 8.0 to 8.1 ± 0.9) and from 8.0 ± 0.6 to 8.7 ± 0.9 after 72 h of storage.

Table II Effect of storage at 20–22 °C without stirring on platelet ohmetric constants (μm^3)

	Duration of storage			
	0 h	24 h	48 h	72 h
Mode	4.30±0.2	4.30±0.2	4.30±0.2	4.60±0.3
Mean volume	8.0 ±0.6	8.1 ±0.9	8.1 ±0.9	8.7 ±0.9

The volume distribution curves were determined by using a Coulter counter Z Bic coupled with C 1000 analyzer (aperture = 70 µm attenuation ½ opening current ¼ K = 0.336) and Isoton solution (Coultronics, France) as diluent (300±10 mosm). The volumetric constants were then calculated [24].

Table III Effect of storage at 20–22 °C without stirring on ^{14}C-serotonin and ^{3}H-adenosine uptake and release by human platelets

Duration of storage, h	^{14}C-serotonin, % of control		^{3}H-adenosine, % of control	
	uptake	release	uptake	release
0	100	100	100	100
24	112	65	85	90
48	88	32	65	67
72	79	10	60	50
96	50	8	46	39

Experimental conditions were as described in the text (Methods). Results were expressed as percentages of the control (activity at day 0 of storage) and represent the mean values obtained in three experiments

0.86 fig. 3). Platelets stored for 48 and 72 h possess less than 23 and 20%, respectively of D platelets and less than 67 and 48%, respectively of C platelet population (C + D/A + B ratio = 0.47 and 0.32, respectively).

Glycogen synthesis activity of the platelet populations shows also a gradual decrease during the platelet storage for 24–48 and 72 h at 20 °C. However it was observed that the G6P-dependent transglucosylase, the 'D' variant of the enzyme, decreased in the heavy C and D platelets, whereas the activity of the enzyme independent on G6P (the 'I' variant)

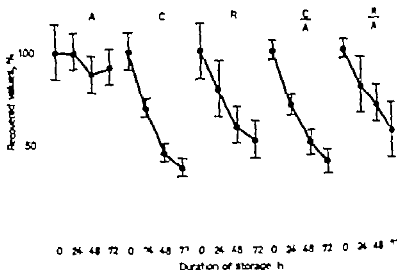


Fig. Effect of storage at 20–22 °C without stirring on the A, C, R parameters in the osmotic shock test (as defined in figure 1). Results (light transmission arbitrary units) were expressed as percentage of the values at day 0 of storage and represent mean values obtained in three experiments.

modified 5HT uptake capacity as compared to the corresponding values of 112%, then, this gradually decreases from 88 to 50% during the platelet storage for 48–96 h (table III). The serotonin release by the 24-hour stored platelets, activated by collagen, is much reduced (65%) as compared to the fresh platelets, and also a very marked decrease of this platelet function (22–8%) was measured after 48 and 96 h of storage. An increased spontaneous release (as measured in the labeled platelets in the absence of the release inducer) of the radioactive material has been noted.

^{3}H-adenosine uptake by platelets decreases after 24 h at 20–22 °C (88%) and the decrease continues and attains 46% at 96 h of storage (table III). The release of the labeled material decreases progressively but is less marked than the serotonin release.

Platelet populations. The mean values for the four normal human platelet populations, isolated from fresh PRP, are: lighter platelets A = 16.08 ± 4%, and B = 18.47 ± 7%, 38.25 ± 4% and 27.67 ± 4% for the heavier C and D populations. The C + D/A + B ratio is thus 1.91. During the PRP storage at 20 °C, the platelet population pattern presents an important modification. After 24 h of storage a decrease of 47 and 17% for the D and C platelets, respectively, was noted (C + D/A + B ratio =

Table IV Glycogen synthesis in the four platelet populations (A, B, C, D) during the platelet storage at the ambient temperature without stirring

Duration of storage, h	Glycogen synthetase, % of control							
	'D' variant				'I' variant			
	A	B	C	D	A	B	C	D
0		100				100		
24	45	38	58	60	46	58	42	52
48	38	44	44	40	40	55	50	46
72	36	40	46	28	42	35	46	40
96	32	40	37	19	42	56	45	40

The 'D' and 'I' variants of the platelet transglucosylase have been determined on platelet populations isolated as described in an assay mixture, containing in 150 μl final volume 0.2 μmoles UDPG, 15,000–20,000 cpm UDP ^{14}C-G, 4 μmoles glycyl-glycine buffer pH 7.4, 2 mg glycogen as 'primer' 0.5 μmol G6P (or buffer) and 100 μl platelet suspension (100–200 μg protein). After incubation at 37 °C for 20 min the labeled glycogen was isolated, purified, solubilized in bidistilled water and used for the radioactivity counting [29]. The transglucosylase-specific activity (cpm mg protein^{-1} h^{-1}) of each fraction was expressed as percentages of the total in the four fractions. Results represent mean values obtained in three experiments and are expressed as percentages of the maximum measured at day 0 of storage.

perimental conditions. Morphological changes, associated with a significant loss of platelet functional and metabolic properties, have been reported and particular emphasis is placed on the important decrease of platelet survival [1, 2, 6, 9, 12, 17]. Even after platelet storage at 20–22 °C, it appeared [19–21, 27, 28] that the bulk of platelet functions as measured by *in vitro* tests was decreased. However, *in vivo* studies indicated a better preservation of platelet hemostatic properties at ambient temperature, with more or less gentle agitation, than at 4 °C.

In our experimental studies, platelets stored at 20–22 °C without stirring in homologous hypercitrated plasma, presented a loss of 2–3% from the initial platelet count, platelets retained a practically normal cell volume repartition and presented no significant change of the PF3 activity as also of the total UV-absorbing material amount.

In addition, the 24-hour stored platelets presented a moderate modification of their capacity to take up ^{14}C-serotonin and ^{3}H adenosine. Both, an active transport and/or a passive diffusion of these molecules across

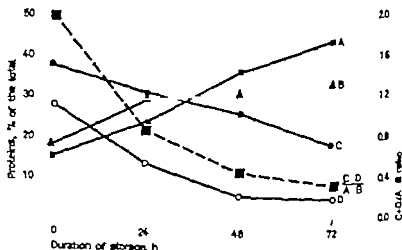


Fig 3 Platelet population changes during platelet storage at 20–22 °C without stirring. Each point represents mean values from three experiments. Platelet populations were separated by ultracentrifugation of platelets in plasma on discontinuous sucrose density gradients (35%, 70% g/V d_{20} = 1.24–1.45) for 60 min at 50,000 g and at 5 °C in an SW 50 rotor of a Beckman L2 centrifuge. The protein content of the four individual platelet fractions A and B, the lighter platelet populations; C and D the heavier platelet populations, and the C + D/A + B ratio have been checked by light absorbance at 280 nm with a Zeiss PMQ2 spectrophotometer. The amount of protein in each fraction was expressed in percent of the total and values at day 0 of storage were assigned as 100%.

decreased, but is still retained by the stored platelets at a higher level than the D variant even after 48 and 72 h of storage at 20 °C (table IV).

Transfusion results. Table V shows some of the results obtained after the transfusion of platelets stored for 24 h at 20–22 °C into thrombocytopenic patients. The patients presented minor hemorrhagic syndromes without septicemic signs or splenohepatomegaly. The platelet counting was performed 1 h after the transfusion. The results in table V show the presence of 25–42% of recirculating platelets from the total platelets administered, except when circulating antibodies were detected. In one case (not presented) a 60% recirculation was obtained. Posttransfusional accidents were never noted.

Discussion and Conclusion

In the past decade there has existed a great number of studies which deals with the field of platelet storage at low temperature in various ex

over platelet population patterns, studied as a part of a comprehensive survey of the global platelet functional and metabolic capacities [26] showed that the presence of approximately 50% of the D population and 80% of the C population of cells seem to be required in order to obtain 25–42% recirculating platelets from the totality of the 24-hour stored platelets administered into thrombocytopenic patients. In addition, these platelets possess a decreased but not completely abolished transglucosylase activity and particularly the 'I' variant of the enzyme which is active in the absence of G6P.

MURPHY and GAARDNER [20] have noted the ability of platelets, stored at the ambient temperature, to recover an elevated level of glycogen after their transfusion. These and our data are suggestive of a reactivation of the glycogen synthesis in the stored platelets when they circulate in a renewed plasmatic milieu. Thus, if the membrane status is certainly important, the complex intracellular metabolic events could interfere with the multiple platelet function and determine the platelet behaviour.

In conclusion, as platelet stored in homologous hypercitrated plasma at 20–22 °C without agitation for 24 h (at least) are able to recirculate in a proportion of 25–42% from the total platelets transfused into thrombocytopenic patients, and no bacterial contamination (as shown by 300 bacteriological examinations performed on various batches of stored platelets) nor posttransfusional accidents were noted, platelets stored in the indicated conditions represent presently a mean to respond to the increasing clinical demands for platelet transfusion.

Acknowledgment

This investigation was supported in part by a grant from The National Institute of Health and Medical Research (INSERM – CRL 74 4 061 1).

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Table I Data obtained following the transfusion of platelets stored for 24 h at 20-22°C without stirring into thrombocytopenic patients

Patient	Diagnosis	Transfused platelet $\times 10^{11}$	Platelet count $\times 10^3$		Circulating platelets %[1]
			before transfusion	after transfusion	
BL	idiopathic aplasia	5	0	45	39
RI	idiopathic aplasia	6	15	20	5[2]
B.	ALL	3	15	45	42
R.	AML	4.7	15	45	27
B.	ALL	4	30	90	25
J	ALL	5.8	25	60	29
CAB	AML	8.4	2	15	5

Circulating blood volume was calculated according to HOPPER *et al.* [14].
[1] Of the platelets administered
[2] Presence of circulating antibodies, determined by the lymphotoxicity test.

the platelet membrane could be operative at least during the first 24 h of storage. The absence of significant changes in the amount of platelet total UV-absorbing material does not exclude a fall in the platelet ATP content, as recently reported by FILIP *et al.* [9]. Indeed, these authors demonstrated that the decrease of the nucleotide was associated with the conversion of ATP in the metabolic adenine nucleotide pool to hypoxanthine during the platelet storage for 72 h either at 4 or 22 °C.

In contrast with the results obtained in the tests precedingly cited, it appeared clearly that only platelets stored up to 24 h (eventually 48 h) retained the ability to be activated by the physiological platelet aggregation-promoting agents. In conjunction with this observation, a significant loss of platelet ability to release the ^{14}C labeled serotonin and the radioactive material after the ^{3}H-adenosine platelet labeling, as also the decrease of their ability to reversibly respond to the osmotic shock, have been observed. Thus, it appears that some of the platelet functions that depend on the membrane integrity [12], such as aggregation, release of intracellular constituents, and the response to the osmotic shock, are better and earlier illustrated by the results obtained in these last tests than in the former ones which also allow to measure changes, but at a moment when less viable cells are present, which are able to assure hemostasis. More-

over platelet population patterns, studied as a part of a comprehensive survey of the global platelet functional and metabolic capacities [26] showed that the presence of approximately 50% of the D population and 80% of the C population of cells seem to be required in order to obtain 25–42% recirculating platelets from the totality of the 24-hour stored platelets administered into thrombocytopenic patients. In addition, these platelets possess a decreased but not completely abolished transglucosylase activity and particularly the I variant of the enzyme which is active in the absence of G6P

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In conclusion, as platelet stored in homologous hypercitrated plasma at 20–22 °C without agitation for 4 h (at least) are able to recirculate in a proportion of 25–42% from the total platelets transfused into thrombocytopenic patients, and no bacterial contamination (as shown by 300 bacteriological examinations performed on various batches of stored platelets) nor posttransfusional accidents were noted, platelets stored in the indicated conditions represent presently a mean to respond to the increasing clinical demands for platelet transfusion.

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versibly in the oxidized state. Such abnormal haemoglobins are called haemoglobins M. In the recessive form, a homozygous deficiency in NADH diaphorase results in methaemoglobinaemia: we will refer to this condition as congenital enzymopenic methaemoglobinaemia (CEM). We recently observed three brothers presenting this disorder. A family study was done subsequently. Because of several interesting aspects we thought it worthwhile to report our cases.

Methods

Blood was stored at 4 °C for periods of between 3 and 36 h before being analysed.

Methaemoglobin was measured according to the method of EVELYN and MALLOY [8] and in some cases according to the method of KIESE [18].

NADH diaphorase activity determinations were made according to HEGESH *et al.* [10]. Units are expressed as nmol/min/mg for RBC and nmol/min/mg protein for WBC. Proteins were estimated by the method of LOWRY *et al.* [21]. NADH diaphorase electrophoresis was performed following the method of KAPLAN and BEUTLER [15], as modified by HOPKINSON *et al.* [12]. Red cells were separated according to age by centrifugation, as described by HERZ *et al.* [11]. NADH diaphorase was subsequently assayed in the 10% 'oldest' and 'youngest' cells thus obtained.

Haemoglobin spectroscopy was performed with a Unicam SP800 spectrophotometer on the deoxy-, oxy-, met- and cyanmethaemoglobin forms. Haemoglobin was further studied by isoelectric focusing in polyacrylamide gel, as described by DRYSDALE *et al.* [7]. The oxygen dissociation curve was determined on whole blood following the method of CLERBAUX *et al.* [4]. 2,3-Diphosphoglycerate(2,3-DPG) was assayed according to NYGAARD and RORTH [24].

Case Reports

Figure 1 illustrates the pedigree of the family reported, in which there was no consanguinity. All known ancestors were of local origin, except the maternal grandfather of subject I, who was born in Spain. Only patients III, III and III were evidently cyanotic.

Patient III (27 years old)

After an uneventful pregnancy and delivery, marked cyanosis was noted at birth, and was ascribed to congenital heart disease. The cyanosis persisted but was well tolerated. At the age of 10, however, the patient complained of easy fatigability. Methaemoglobinaemia was then diagnosed and treated with vitamin C, 1,000 mg daily. The cyanosis improved markedly. Finger clubbing was noticed for the first time at the age of 18. Six years later, the patient consulted our department because

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Congenital Enzymopenic Methaemoglobinaemia

Clinical and Biochemical Study of a Family with Three Homozygotes

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Key Words Haemoglobin Methaemoglobinaemia NADH diaphorase deficiency Osteoarthropathy

Abstract Three brothers with congenital recessive methaemoglobinaemia without mental retardation were found to be homozygous for NADH diaphorase deficiency Twelve family members were heterozygous. One of the probands had marked digital clubbing, an unusual feature in this disease. In the probands, the red cell NADH diaphorase was very low and unstable, whereas in the leucocytes the enzyme was present at a normal level. Isoelectric focusing of haemoglobin in the three probands showed that the α-chain was preferentially oxidized spontaneously This confirms the greater oxidizability of this chain, as already described on isolated chains.

Methaemoglobin is formed when the iron atoms of haemoglobin are in the oxidized, ferric state Methaemoglobin is unable to participate in oxygen exchange. Haemoglobin is continuously being oxidized into methaemoglobin, but in the normal individual an intra-erythrocytic reducing system consisting mainly of NADH diaphorase keeps the concentration of methaemoglobin at a low level about 1% of the total haemoglobin. Higher concentration will give a chocolate brown colour to the blood and a slate grey cyanotic aspect to the affected patient.

Methaemoglobinaemia is acquired (toxic methaemoglobinaemia due to a number of drugs or chemicals increasing the rate of oxidation of haemoglobin) or much more rarely familial and congenital with either a dominant or a recessive mode of inheritance In the former a structural anomaly of one of the haemoglobin chains causes the molecule to remain irre-

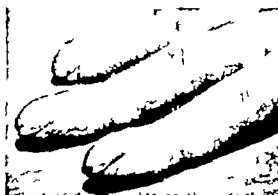


Fig. 2. Pronounced digital clubbing in patient III



Fig. 3. Isoelectric focusing on patient III and case II before ( ) and after (**) reduction with KCN.

ioelling. After treatment with KCN, the methaemoglobin and intermediate bands disappeared (fig. 3). The injection of 100 mg Methylene Blue, induced drop of the methaemoglobin level from 10 to 1.3% than 20 min. The basal level of only 10% was explained by the ingestion of vitamin C 500 mg 3 days prior to the test. A rise in the P_{50} was observed 60 min after injection (table I) then the methaemoglobin level was 3.6%. Under maintenance therapy of vitamin C 1,000 mg and methylene blue 100 mg daily the subjective symptoms improved markedly and the methaemoglobinaemia was 8%

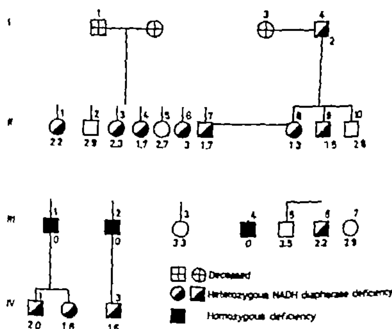


Fig. 1. Pedigree of the family. The figure above the symbol is the serial number for a given generation; the figure underneath the symbol gives the NADH diaphorase activity in nmol/min/mg Hb. Normal values 3 ± 0.5; values lower than 2.4 were considered as heterozygous.

of dyspnea, dizziness, palpitations and precordial pain, not typically related to exercise. He had taken vitamin C irregularly during the previous months. Physical examination revealed marked slate grey cyanosis and digital clubbing (fig. 2), but was otherwise entirely negative. In particular there was no mental retardation, nor were there neurologic symptoms.

RBC 5,440,000/µl, haemoglobin 17.3 g%, haematocrit 54.1%. A blood smear was normal. Arterial blood gases were normal: pH 7.36, pCO_2 40 mmHg, pO_2 91 mmHg and bicarbonate 24 mmol/l. Other routine blood tests were negative. A chest roentgenogram and an electrocardiogram were normal. An exercise electrocardiogram on the bicycle ergometer was negative. Arterial pO_2 after 20 min of pure oxygen breathing was 630 mmHg; this indicated there was no pathologic right left shunt [5]. Methaemoglobinaemia was 29%. NADH diaphorase activity in RBC, WBC, and in young and old RBC are given in table I. NADH diaphorase electrophoresis showed a weak band with normal mobility.

Spectroscopic study of the haemoglobin was strictly normal, hereby excluding an M-type abnormal haemoglobin. The oxygen dissociation curve, the partial oxygen pressure needed for a 50% haemoglobin saturation (P_{50}), and the 2,3-DPG concentration were normal (table I). Isoelectric focusing of haemoglobin revealed an oxyhaemoglobin band and a weak methaemoglobin band; midway between them and close to each other were two intermediate bands: IB_I and IB_{II}, the latter predom-

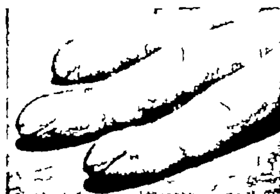


Fig. 2. Pronounced digital clubbing in patient III

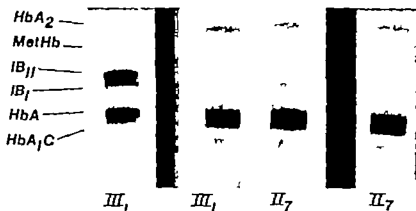


Fig. 3. Isoelectric focusing on patient III and case II before ( ) and after ( ) reduction with KCN.

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Table I Pertinent laboratory findings in the three patients (III_1 III_2 III_4) and three other family members (cases I_4, II_7 II_8)

	Normal values	Case I_4	Case II_7	Case II_8	Patient III_1 methylene blue		Patient III_2 methylene blue	
					before	after	before	after
Methaemoglobin % total Hb	< 1.5	1.1	0.4	0.5	10	3.7	11.4	7.4
NADH diaphorase								
In RBC	3 ± 0.5	?	1.7	1.4	0	–	0	
In 'young' RBC		–	–	–	0.5	–	0.8	
In old RBC			–	–	0	–	0	
In WBC	16	–	18	16	18	–	19	
P_{50}, mmHg	26.3 ± 0.5		–	–	26.5	29.0	26.5	29.8
2, 3 DPG µmol/g Hb	13.8 ± 2.7	–	–	–	14	15.2	14.4	11.8

[1] Determined simultaneously and under the same conditions on a control subject.
The NADH diaphorase activity is expressed as nmol/min/mg Hb for RBC and nmol/min/mg prot for WBC.

During a more than 2 year follow-up he again experienced intermittently similar symptoms although to a lesser degree, but his symptoms now seemed to be related partly to psychic factors. The digital clubbing remained unchanged.

Patient III (24 years old) and Patient III (15 years old)

These patients also presented congenital cyanosis, diagnosed as methaemoglobinaemia at age 7 and at birth respectively. They complained only of cosmetic problems related to the cyanosis, which, however was greatly improved by vitamin C. Apart from the cyanosis, the physical examination was unremarkable in both. A slight erythrocytosis was found in patient III RBC 5 470,000/µl, haemoglobin 17.5 g%, haematocrit 52%. In patient III_4 the same values were 4 910,000, 15.4 and 4_6, respectively. Pertinent laboratory findings are given in table I. Haemoglobin spectroscopy was normal in both subjects the isoelectric focusing was essentially the same as in case III. In patient III_2, 100 mg i.v. methylene blue was given the methaemoglobin level dropped from 11.4 to 1.6% after 20 min and 7.4% after 60 minutes, at this time a rise in the P_{50} was observed (table I).

Other Family Members

None of these subjects looked cyanotic. The results of pertinent laboratory investigations are given in table I and figure 1

Discussion

CEM is a rare disease, JAFFÉ and HSIEH [14] found approximately 260 patients in the literature. This family studied over four generations, confirms very well the autosomal recessive mode of inheritance of CEM. Only the homozygotes with no detectable NADH diaphorase activity had an appreciable amount of methaemoglobulin, and hence were cyanotic.

In the three patients, the enzyme seemed particularly unstable *in vivo* whereas some residual activity could be found in the youngest erythrocytes, virtually none was detected in the oldest (table I). An attempt of purification of the enzyme in patient III was unsuccessful, indicating that the enzyme was also very unstable *in vitro*

As can be inferred from electrophoretic studies, CEM is a heterogenous disease: enzyme variants with decreased activity and normal [15] faster [15] or slower [1] mobility have been described. Electrophoretic variants with normal activity also have been reported [6] In some cases, no enzyme activity at all can be detected on electrophoresis [15] These findings indicate that multiple structural anomalies of the enzyme are possible, the enzyme function being hereby destroyed, reduced, or unaffected. In patient III a weak band with normal mobility was demonstrated.

Approximately 10% of the cases of CEM are associated with severe mental retardation and neurological signs such as athetosis leading to death in childhood. Three non related children [16, 17 20] presenting this association were shown to have a markedly reduced erythrocytic and leucocytic NADH diaphorase activity whereas in seven other non-related enzyme-deficient homozygotes without mental retardation, only the RBC were deficient [17] The finding of normal leucocytic NADH diaphorase activity in our cases, unassociated with cerebral symptoms, is in agreement with the above-mentioned observations.

Patient III presented several symptoms which we considered to be related to his high methaemoglobin level. Although CEM patients usually tolerate their disease perfectly well, levels higher than 30% may be associated with symptoms such as dyspnea and headache [13] Patient III also had a pronounced digital clubbing, which appeared progressively at age 18. There was no associated cardiopulmonary disease nor were there

After the present study was completed, we observed two additional cases with mental retardation and NADH diaphorase deficiency in the leucocytes, and three other cases without mental retardation and without enzyme deficiency in the leucocytes [LEROUX and KAPLAN, unpubl. results].

any of the other conditions (biliary cirrhosis, regional enteritis, ulcerative colitis, pituitary and thyroid disease...) clubbing is occasionally associated with [9]. Thus clubbing was considered to be either idiopathic or related to CEM. Since idiopathic clubbing can occur at or after puberty [9], the onset at the age of 18 is in favour of this possibility. Furthermore, in the main review articles about CEM [13, 14] clubbing is said not to occur in association with this disease. However MAUER [23] states, although without presenting references or documented cases, that chronic methaemoglobinaemia is one of the causes of digital clubbing. This sign has also been described in a 6-year-old patient with CEM [2]. Since this child also had lung tuberculosis, a disease known to be a possible cause of clubbing [9], the aetiological role of methaemoglobinaemia did not seem to be definitely established.

Patients III_1 and III_2 had a slight erythrocytosis. This has occasionally been described in CEM [13, 14] and is ascribed to the loss in oxygen carrying capacity due to the methaemoglobin. Furthermore, the lowered P_{50} found in some cases of CEM [13], but not in our patients, can be an additional factor inducing erythrocytosis. *In vitro* and *in vivo* experiments on man and dog blood, respectively, have shown methaemoglobin to increase the oxygen affinity of the remaining haemoglobin and, thus, lower the P_{50} [13]. Accordingly, in some cases of CEM a decrease of the P_{50} is observed, whereas in other cases, as in patients III_1 and III_2, a normal P_{50} is demonstrated [13]. In our cases, the decrease of the methaemoglobin level by methylene blue results in a rise of the P_{50} from a normal to a supranormal value (table I) in spite of normal 2,3-DPG concentrations. This finding may point to the presence, in patients III_1 and III_2, as well as in other cases of CEM with a normal P_{50}, of a P_{50} rising factor other than 2,3-DPG neutralizing the P_{50} decreasing effect of methaemoglobin.

As already stated, in the three patients the isoelectric focusing revealed, midway between the oxyhaemoglobin and the weak methaemoglobin band, two intermediate bands, IB_I and IB_{II}, the latter predominating (fig. 3). BUNN and DRYSDALE [3] found a similar pattern in partially oxidized haemoglobins; however, they found IB_{II} to be predominant only at pH 6.6 whereas at pH 7.1 and 7.5 the reverse was observed. They demonstrated spectroscopically that the two IB were each half oxidized, corresponding to $\alpha_2\beta^+_2$ and $\alpha^+_2\beta_2$, but did not determine which of the two compounds each of the IB corresponded to. A recent observation by one of us (R. K.) and other workers establishes that IB_{II} is $\alpha^+_2\beta_2$ [19]. Isoelectric focusing was performed on the haemolysate of a

patient with methaemoglobinaemia due to haemoglobin M Boston, where the structural anomaly is in the α-chain. Obviously the methaemoglobin in this subject had to be $\alpha^{+}{}_{2}\beta_{2}$ and on focusing it was shown to correspond to IB_{II}. Our findings indicate that in CEM the α-chain is more susceptible to oxidation and confirm the observation of MANSOURI and WINTERHALTER [22]. These authors demonstrated, on isolated chains obtained after partial oxidation of haemoglobin that the α-chain oxidized at a rate ten times faster than the β-chain and ascribed this to a greater oxidizability of the α-chain. Theoretically however a lesser reducibility of the α-chain by the NADH diaphorase could have been involved as well. Since in our patients, no enzyme was present, the predominance of $\alpha^{+}{}_{2}\beta_{2}$ over $\alpha_{2}\beta^{+}{}_{2}$ can only be due to a greater oxidizability of the α-chain, as postulated by MANSOURI and WINTERHALTER.

Acknowledgements. We are indebted to Dr. T. CLERBAUX (Laboratoire d'exploration fonctionnelle cardio-pulmonaire, Université Catholique de Louvain, Belgium) who kindly performed the oxygen dissociation curve and 2,3-DPG determination.

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A Case with Both Hemoglobins C and N-Baltimore

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Key Words. Hb C · Hb N-Baltimore · Hemoglobinopathies

Abstract. Hemoglobins C and N-Baltimore were detected in a 75-year-old black man. Although mild anemia and abnormal erythrocyte morphology resembling thalassemia were present, detection of this combination of hemoglobins at an advanced age suggests that it does not limit normal activities.

The β-chain abnormal hemoglobin in which glutamic acid replaces lysine at residue 95 is now known as Hb N-Baltimore [4] but has also been called N Memphis [10], Jenkins [5], Hopkins-1 [7], and Kenwood [8]. The substitution in Hb N-Baltimore is one of the two substitutions in the β-chain of Hb Arlington Park [1] which also has the same substitution as Hb C. This paper reports on an individual who had Hb N-Baltimore in combination with Hb C.

Case Report

E.D., a 67-year-old black man, was first seen in 1966 with acute bronchitis, mild chronic obstructive lung disease, congestive heart failure, and mild anemia. A past history of peptic ulcer disease was obtained, and he had been a heavy smoker. There was no prior history of anemia in the patient nor in his family.

There were subsequent hospital admissions for pneumonia in 1968 and acute bronchitis in 1970. In 1969 and 1970, he suffered acute myocardial infarctions. In 1973, a mass in the right middle lobe was noted. This mass slowly enlarged and was associated with cachexia and abnormal liver function tests. Bronchogenic carcinoma was assumed, and as the patient refused invasive procedures, he was treated supportively.

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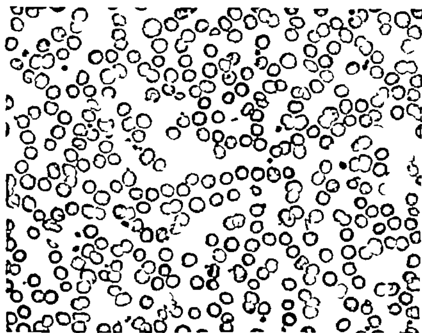


Fig 1 Peripheral blood smear Wright stain, showing target cells associated with moderat hypochromasia and microcytosis similar to thalassemia.

sis at pH 6.2. The hemoglobin with the behavior of Hb C was not characterized further but chemical identification was made on the other hemoglobin which had the approximate behavior of an Hb N By DEAE Sephadex chromatography the quantity of Hb C+Hb A_2 was 40⁰/ and of the Hb N was 60⁰/₀.

Although the Hb A_2 is coincident with the Hb C in the electrophoresis (fig. 2) the absence of an abnormal Hb A_2 suggested that the abnormality of the Hb N was in the β-chain. When amino acid analysis of the separated chains of the Hb N was made, the analysis of the β-chain suggested the replacement of a lysyl by a glutamyl (or glutaminyl) residue. All tryptic peptides except βT 12b were isolated and identified by amino acid analysis, but it was evident that the expected peptides βT 10 and βT 11 were now joined because of the exchange of a glutamyl (or glutaminyl) for a lysyl residue. Table I shows the analysis of βT 10, 11 after purification by Dowex 1 chromatography [12] in comparison to the

The anemia was persistent and at no time was jaundice or hepatosplenomegaly noted. In 1974 hematologic evaluation was undertaken. Six weeks later he died of respiratory failure and pneumonia at age 75. Autopsy was refused.

Materials and Methods

Blood was collected in Vacutainer tubes with EDTA as anticoagulant. Hematological investigation was made by standard methods.

Hemolysates were prepared by lysing the washed cells with 1.5 vol of water and 0.4 vol of CCl_4. After double centrifugation, the clear hemoglobin solution was used for various experiments. Electrophoresis was made at alkaline pH on paper and starch gel and at acid pH on citrate-agar. Chromatography on DEAE-Sephadex [6, 9] was used for both analytical and preparative purposes. Hemoglobin chains were separated by countercurrent distribution [2] and aminoethylated. Tryptic digestion was done at pH 8 and 40 °C for 4 h; enzyme at a 1:200 enzyme: substrate ratio was added at 0 and 2 h. The soluble tryptic peptides were separated as previously described [11]. Amino acid analyses were made with a Beckman amino acid analyzer after 24 h hydrolysis in 6 N HCl *in vacuo* at 110 °C.

Results

The mean Hb was 11.7 g%, (range 10.0–13.5 g%), the mean PCV was 37% (range 32–44%) and the mean red cell count was 5.34×10^6/μl (range $4.76–5.81 \times 10^6$/μl). The MCV was 69 μm³, MCH 21.9 pg and MCHC 31.6%. The reticulocyte index was 1.7%. A sickle cell preparation and a glucose-6-phosphate dehydrogenase spot test were normal. The total bilirubin ranged from 0.2 to 0.6 mg/dl. The serum iron was 28 μg/dl and the TIBC 291 μg/dl.

The red cell morphology showed moderate anisopoikilocytosis, marked targeting, moderate hypochromia and microcytosis, and slight polychromasia (fig. 1). A bone marrow aspirate revealed 55% cellularity, mild erythroid and megakaryocytic hyperplasia. Iron stores were increased. 8% of the normoblasts had siderotic granules with no ringed sideroblasts. No tumor cells were seen.

Upon starch gel electrophoresis at pH 9.0, the hemoglobin showed the pattern in figure 2: one hemoglobin was at the position of Hb C (or Hb A_2) and the other was approximately as far ahead of Hb A as Hb S is behind. On the paper electrophoresis, the two components were equally ahead of and behind Hb A. On the other hand, the two hemoglobins were in the positions of Hb C and Hb A after citrate agar electrophore-

Table I. The amino acid composition of peptide βT 10, 11 of the Hb N and peptides βT 10 and βT 11 of Hb A in terms of residues per peptide

Amino acid	β^{N}T 10, 11 (analysis)	β^{A}T 10 (expected)	β^{A}T 11 (expected)
Lys		1	
AE-Cys	0.80	1	
His	1.88	1	1
Arg	0.94		1
Asp	2.98	1	2
Thr	1.93	2	
Ser	1.08	1	
Glu	3.05	1	1
Pro	0.91		1
Gly	1.12	1	
Ala	1.19	1	
Val	1.06		1
Leu	2.97	2	1
Phe	1.97	1	1

prevent him from leading a normal life until 75 years. Relatively minimal effects from the combination of Hb C and Hb N-Baltimore are perhaps not unexpected. Hb N-Baltimore has lesser interaction with Hb S than does Hb A [3]. However, because the tendency of Hb C to crystallize differs from the gelling of Hb S, there may be interactions of Hb C and Hb N-Baltimore that would be evident only from experiment. This case is an example of the individual in whom homologous crossing-over might produce Hb Arlington Park as suggested by ADAMS and HELLER [1]. None of E. D.'s family was available for study.

Acknowledgements. This work was supported in part by grant (HL-02558) from the National Institutes of Health, US Public Health Service and Sickle Cell Center grant HL 15162. We thank BARBARA ATKINSON, LESLIE E. AMS, JAMES JARWAY and ROGER SHELTON for technical assistance.

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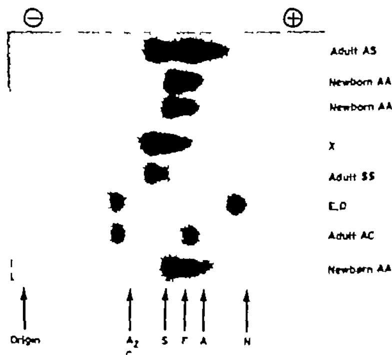


Fig 2 Starch gel electrophoretic pattern of hemoglobin after electrophoresis at pH 9 for 4 h at 25 V/cm *o*-Dianisidine stain X is unrelated to this study

expected βT 10 and βT 11 Consequently it may be concluded that residue β95 has been replaced by a glutamyl or a glutaminyl residue.

Paucity of material prevented us from distinguishing between glutamic acid or glutamine in residue 95 of the Hb N CLEGG *et al* [4] used electrophoretic behavior of Hb N Baltimore to decide that residue 95 is glutamic acid. DOBBS *et al* [5] reached the same conclusion because of the identity of electrophoretic behavior in Hb N Baltimore and Hb Jenkins. To the extent that electrophoretic behavior can be compared Hb N of our case behaves like Hb N Baltimore and Hb Jenkins.

Discussion

Although the erythrocytes had abnormal morphology and mild anemia was present during the period of observation in the patient's advanced years, the unusual combination of hemoglobins apparently did not

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His	1.88	1	1
Arg	0.94		1
Asp	2.98	1	2
Thr	1.93	2	
Ser	1.08	1	
Glu	3.05	1	1
Pro	0.91		1
Gly	1.12	1	
Ala	1.19	1	
Val	1.06		1
Leu	2.97	2	1
Phe	1.97	1	1

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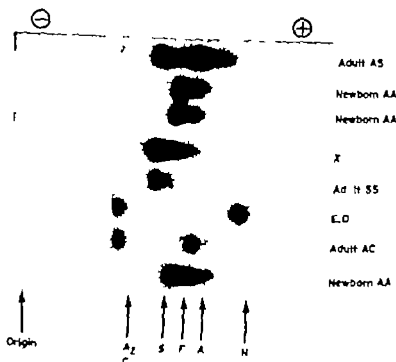


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Monoclonal Gammopathy of IgA and IgE Type in a Case of Chronic Lymphatic Leukaemia

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Key Words. Gammopathy · Lymphatic leukaemia · Paraproteins

Abstract. A 70-year-old woman was hospitalized with chronic lymphatic leukaemia. In the blood drawn during the first period of hospitalization, monoclonal IgA was detected. At the second hospitalization, 4 months later, monoclonal IgE was found whereas the IgA paraprotein was no longer detectable. Both monoclonal immunoglobulins were of the κ-light-chain type. Rapid deterioration and death prevented further investigation.

The appearance of a homogeneous fraction in the region of the immunoglobulins points to an antibody-producing clone which is represented by an excessively high number of cells. This is typical for plasmocytoma. Monoclonal immunoglobulins, however, are also often seen in other diseases like carcinoma, chronically progressive inflammatory processes and sometimes in apparently healthy persons. The frequency of gammopathies is decreasing in respect to the classes IgG, IgA, IgM, IgD and IgE. As a consequence, monoclonal immunoglobulins of the IgE class are very seldom and have been detected as the last group (1,2). Now another IgE paraprotein has been found in a patient who also had an IgA paraprotein.

Case Report

The patient, a 70-year-old woman, was admitted to another hospital[1] because of extreme paleness, weakness and weariness. She was treated previously by her physician for hypertension. In 1971, she suffered an episode of herpes zoster. During the last months before hospitalization an aggravating anaemia was striking.

[1] I thank Prof. Dr. A. Wagner, Director of the hospital of Coburg (FRG), who let us have the clinical data.

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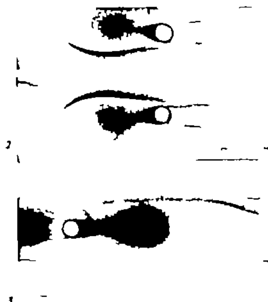


Fig. 2. In both wells patients serum, in the trough monovalent anti-IgE serum

Fig. 3. In the central well patients serum, in the troughs monovalent anti- -serum (above) and anti-λ-serum (below).

antisera, the light chain type was identified by using monovalent antisera (fig. 3). Isolated light chains were not detected. All immunoelectrophoretic tests showed the same immunoglobulin aggregates. The serum level of IgE measured by the radioimmunoassay (Phadebas Pharmacia) was 260 µg/ml

Discussion

The physician already supposed that the symptoms were due to a malignant disease of the lymphatic system. In the hospital, this suspicion was confirmed. Although there was no hint for plasmocytoma or paraproteinaemia, the detection of the monoclonal IgA was not contradictory (this is the reason why we did not document immunoelectrophoresis with a picture). But we were surprised by the immunoelectrophoretic pattern of the serum 4 months later. By then the monoclonal IgA had vanished. However, a deposit of immunoglobulins in the agar was conspicious and so we analyzed the serum in detail finding the monoclonal IgE after additional tests. On the one hand, no phenomena hinted at the

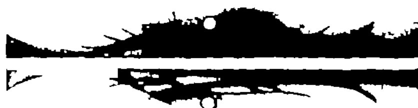


Fig 1 In the central trough polyvalent anti-human serum, in the wells patient's serum (above) and a control serum (below)

The results of the tests performed at that time were as follows. sedimentation rate 125 mm/h haemoglobin 7.3 g/100 ml erythrocytes $2\times10^6/\mu l$ leucocytes 2,200/ l differential count 2% stab cells, 29% neutrophils, 67% lymphocytes, % monocytes. Thrombocytes 47 000/ l The other blood and urinary chemistry data were normal The tentative diagnosis of the physician was haemoblastosis or plasmocytoma.

The first examination at hospitalization showed an advanced anaemia, beginning pretibial oedema and dyspnoea. The margin of the liver was 5 cm below the costal arch spleen or lymph nodes were not palpable. Total protein 6.1 g/100 ml electrophoresis albumins 66%, α-globulins 2%, α_2-globulins 8%, β-globulins 6% γ-globulins 18%. Coombs test, negative Bone marrow aplastic syndrome with atypical round cells, probably lymphocytes.

An IgA of monoclonal origin was detected by immunoelectrophoresis. By application of monovalent antisera (Behringwerke) κ-light-chain type was identified. IgG was diminished, IgM was normal. The patient was allowed to go home and she had to be hospitalized once more 4 months later The examination showed the same results, spleen and lymph nodes were not palpable. Sedimentation rate 183 mm/h haemoglobin 2.1 g/100 ml erythrocytes 700,000/ l leucocytes 4600/µl differential count lymphocytes 99%, 1 atypical cell All other tests proved normal. Total protein was 6.2 g/100 ml electrophoresis albumines 53%, α-globulins 4%, α-globulins 13%, β-globulins 9%, γ-globulins 21 %. Osteolytic lesions were not detected by X-ray examination. Treatment with steroids, antibiotics and fresh blood could not prevent rapid deterioration of the patient's condition. Some days later she died. An autopsy was not performed.

During this second hospitalization, another serum sample was sent to us. Contrary to the first sample no monoclonal immunoglobulin could be identified with certainty after application of a polyvalent anti-human serum. However a nonspecific precipitate consisting of immunoglobulins was seen in the agar (fig. 1). It was placed from the start into the direction of the cathode After applying monovalent antisera, a clear line was seen in the agar (fig.) IgG was diminished, IgM was normal IgA was not found any more After absorption of IgG and IgM with

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Proliferation Characteristics of Lymphoid Cells of Human Bone Marrow in Health and in Chronic Renal Failure[1]

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Key Words. Autoradiography · Bone marrow · Cytophotometry · DNA synthesis · Lymphoid cells · Renal anaemia

Abstract. Proliferation of bone marrow lymphoid cells of 8 healthy subjects and 7 patients with chronic renal insufficiency was compared using the cytophotometric measurement of the DNA content combined with ^{3}H-thymidine labelling *in vitro*. In renal insufficiency the cells showed a marked decrease of the proliferation activity. This is apparent from the impaired labelling indices and accumulation of diploid and unlabelled cells. The data suggest that the lymphoid cell proliferation is affected by the same pathophysiological mechanism as that leading to impairment of red cell production. By subclassification according to cytoplasmic basophilia, this proliferation disturbance could be related to the basophilic and not to the pale type of lymphoid cells.

Cytologically in bone marrow early haemopoietic precursors, reticulum cells macrophages, and small lymphocytes can be distinguished from so-called lymphoid cells because they are larger than the typical bone marrow lymphocytes. This cell type shows proliferative activity and is obviously involved in the production of B lymphocytes or plasma cells. Moreover experimental studies proposed that this cell type, for which the term 'transitional cell' was introduced, may as well act as haemopoietic stem cell [7, 9, 16, 17]. In the present study lymphoid cells from healthy subjects and from patients with chronic renal failure were analysed with the cytophotometric determination of the DNA content combined with autoradiography with ^{3}H-thymidine (^{3}H TdR) *in vitro*. The

[1] Dedicated to Prof. Dr. W. Hoffmeister, Mannheim, on the occasion of his 65th birthday.

extremely increased IgE, on the other hand there was no indication for a sensitization either e g infestation with worms. Typically altered plasma cells or osteolytic lesions were not detected.

Double paraproteinaemia as well as the change of the type of the paraprotein occur rarely In this patient, however the same type of light chains is astonishing. Nevertheless, a conexion between both malignant clones is not probable. It is, however remarkable that IgA and IgE producing cells are mainly located in the same region i.e. in the mucosa, thus hinting at a common initiating factor With respect to the change of the immunoglobulins it cannot be excluded that both monoclonal immunoproteins were present in either serum sample, but that in the first sample the IgE was too lowly concentrated to be detected by application of a polyvalent antihuman serum and that in the second sample the IgA may have been precipitated in the agar as a deposit. Moreover it must be suggested that the atypical qualities of the monoclonal IgE caused a wrong result of the radioimmunoassay by nonspecific effects because proteins with a level of 260 μg/ml do not show a clear line as in the present pattern.

Referring the data of the physical examination with hepatosplenomegaly the lacking osteolytic lesions, the blood count and the paraproteinaemia, this disease is based on the malignant transformation of B cells resembling a chronic lymphatic leukaemia Further studies, like identification of B- or T-cell markers of cells taken from bone marrow blood or biopsy for a better classification was not possible due to the rapid deterioration of the patient s condition and sudden death It must be stated, however that the presented case was a malignant disease of the immune system Nowadays not only morphological findings but also functional criteria are the basis for the characterization of such a process. According to them, the patient suffered from a lymphoplasmocytoid immunocytoma, which may also show symptoms of chronic lymphatic leukaemia.

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Table III. Myelograms (%) of 7 patients with chronic renal anaemia

	Case No.						
	1	2	3	4	5	6	7
Basophilic erythroblasts	10.1	7.5	11.2	5.8	7.5	7.4	9.5
Polychromatic erythroblasts	9.5	9.0	18.9	11.0	9.6	5.5	11.4
Orthochromatic erythroblasts	12.2	12.5	23.4	21.6	10.5	5.1	16.3
Erythropoiesis total	31.8	29.0	53.5	38.4	27.6	18.0	37.2
Myeloblasts	0.8	0.5	0.8	0.9	0.4	0.1	0.2
Promyelocytes	3.6	4.8	1.9	3.0	3.0	6.9	2.6
Myelocytes	10.0	10.3	7.0	13.3	11.1	14.8	12.7
Metamyelocytes	7.1	9.4	4.8	6.2	8.9	10.1	6.4
Band neutrophils	15.0	12.9	7.6	12.0	13.3	14.3	13.4
Neutrophils	6.8	5.3	3.3	3.1	12.1	7.2	7.4
Granulopoiesis total	43.3	43.2	25.4	38.5	48.8	53.4	42.7
E:G ratio	0.7	0.7	2.2	1.0	0.6	0.3	0.9
Eosinophils	3.8	1.8	2.6	3.1	4.0	3.6	5.5
Basophils	0.0	0.6	0.2	0.0	0.0	0.3	0.1
Promonocytes	0.9	0.8	0.7	1.2	0.8	1.0	0.7
Monocytes	3.6	2.9	3.4	2.5	2.7	3.7	3.6
Plasma cells	2.8	2.5	3.0	0.8	1.7	3.2	1.7
Blasts	0.9	0.3	0.7	0.4	0.2	0.2	0.0
Histiocytes	0.0	0.3	0.0	0.0	0.3	0.1	0.0
Lymphocytes	10.1	15.1	7.0	8.7	10.5	11.8	7.1
Lymphoid cells	2.8	3.5	3.5	6.4	3.4	4.9	1.4

ther to the diploid nor to the tetraploid range. The lymphocytes always show diploid DNA values and no ^3H-TdR labelling. On the contrary the lymphoid cells show diploid and hyperdiploid DNA values. In comparison with normal values, the proportion of hyperdiploid cells in chronic renal failure is markedly diminished, the proportion of cells in G_1 and G_2 increased and followed by a decrease of cells in S. The subclassification of the lymphoid cells demonstrates, that most of the hyperdiploid and ^3H-TdR labelled cells must be attributed to the basophilic subgroup, both in

Table II Myelograms (%) of 8 healthy subjects

	Subject No.								mean ± SD
	1	2	3	4	5	6	7	8	
Basophilic erythroblasts	7.4	5.4	6.7	8.8	7.8	11.0	10.3	8.1	8.2±1.8
Polychromatic erythroblasts	9.4	9.5	11.8	12.4	11.0	11.4	11.6	12.0	11.1±1.1
Orthochromatic erythroblasts	11.3	13.9	19.2	14.2	15.4	22.5	13.9	17.3	16.0±3.6
Erythropoiesis total	[illegible]8.1	28.8	37.7	35.4	34.2	44.9	35.8	37.4	35.3±6.5
Myeloblasts	0.5	1.5	1.1	0.6	0.8	0.2	0.1	0.[illegible]	0.6±0.5
Promyelocytes	7.2	6.0	3.9	1.8	5.1	2.0	9.5	4.9	5.0±2.6
Myelocytes	9.1	10.7	8.9	6.7	8.6	8.8	6.0	9.7	8.6±1.5
Metamyelocytes	9.6	6.3	6.6	8.[illegible]	5.8	7.0	4.5	5.1	6.6±1.7
Band neutrophils	18.4	13.6	10.4	13.4	16.8	16.8	16.5	17.6	15.4±2.7
Neutrophils	5.3	7.6	4.5	6.5	5.2	3.3	4.1	4.3	5.1±1.4
Granulopoiesis total	50.1	45.7	35.4	37.2	42.3	38.1	40.7	41.8	41.3±4.8
E G ratio	0.6	0.6	1.1	1.0	0.8	1.2	0.9	0.9	0.9±0.2
Eosinophils	1.9	1.4	4.0	3.6	3.3	2.8	3.4	2.4	2.9±0.9
Basophils	0.0	0.1	0.1	0.2	0.2	0.1	0.2	0.3	0.2±0.1
Promonocytes	0.8	0.6	0.8	0.5	0.8	0.5	0.5	0.3	0.6±0.2
Monocytes	3.1	[illegible]4	2.5	1.9	3.2	2.1	3.3	2.2	2.6±0.5
Plasma cells	1.6	1.5	2.2	1.9	1.9	1.0	2.9	1.0	1.8±0.7
Blasts	0.2	0.1	0.2	2.6	0.3	0.1	0.3	0.6	0.6±0.8
Lymphocytes	13.3	18.6	15.8	16.0	12.9	9.4	1[illegible]4	13.3	14.0±[illegible]
Lymphoid cells	0.9	0.8	1.3	0.7	0.9	1.0	0.5	0.7	0.9±0.2

6 megaloblastoid nuclear changes were seen. The percentage of lymphocytes in chronic renal disease was lower than in the healthy control group The lymphoid cells were markedly increased in all patients (table III)

DNA content and ³H TdR-labelling of lymphoid cells. The percentages of diploid and unlabelled (G_0+G_1) ³H TdR labelled (S) and tetraploid and unlabelled (G_2) cells of the different cell groups in normal and pathologic conditions are given in table IV The term U (= unrecognizable) was used for unlabelled cells which could be attributed nei-

Table III Myelograms (%) of 7 patients with chronic renal anaemia

	Case No.						
	1	2	3	4	5	6	7
Basophilic erythroblasts	10.1	7.5	11.2	5.8	7.5	7.4	9.5
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Basophils	0.0	0.6	0.2	0.0	0.0	0.3	0.1
Promonocytes	0.9	0.8	0.7	1.2	0.8	1.0	0.7
Monocytes	3.6	2.9	3.4	2.5	2.7	3.7	3.6
Plasma cells	2.8	2.5	3.0	0.8	1.7	3.2	1.7
Blasts	0.9	0.3	0.7	0.4	0.2	0.2	0.0
Histiocytes	0.0	0.3	0.0	0.0	0.3	0.1	0.0
Lymphocytes	10.1	15.1	7.0	8.7	10.5	11.8	7.1
Lymphoid cells	2.8	3.5	3.5	6.4	3.4	4.9	1.4

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Table IV Cell cycle distribution of bone marrow lymphoid cells in 7 patients with chronic renal anaemia and in 8 healthy volunteers

Subjects	Total lymphoid cells					Basophilic lymphoid cells					Pale lymphoid cells				
	n	G_0+G_1 %	S, %	G_2, %	U	n	G_0+G_1 %	S, %	G_2, %	U	n	G_0+G_1 %	S, %	G_2, %	U
Patients, n=7															
1	111	81	11	7	1	52	69	15	14	2	29	97	3	–	–
2	93	69	19	8	4	47	60	26	10	4	19	68	21	11	–
3	97	92	5	2	1	45	85	11	2	2	36	100	–	–	–
4	108	86	13	1	–	60	80	18	2	–	15	93	7	–	–
5	117	95	3	2	–	55	89	6	5	–	19	100	–	–	–
6	101	97	–	3	–	59	97	–	3	–	11	91	–	9	–
7	106	82	10	8	–	68	82	10	8	–	13	69	8	23	–
Normal subjects, n=8															
Range	100–136	24–58	32–60	7–15	0–2	33–68	11–48	39–69	11–22	0–4	18–36	67–88	12–28	0–5	0
Mean±SD		43±12	44±11	12±3	1±1		29±13	54±12	16±4	1±1		80±6	18±5	2±2	0

n = Number of cells G_0+G_1 = diploid unlabelled cells S = labelled cells G_2 = tetraploid unlabelled cells U = unrecognizable cells.

normal and pathologic condition. The pale cells are mostly diploid in both groups. Therefore, significant differences are only present in the subgroup showing distinct basophilia.

Discussion

The results of the present study may be summarized as follows: in chronic renal anaemia the proportion of bone marrow lymphoid cells seems to be higher than the mean of 0.9% seen in healthy subjects. On the other hand, the proliferation activity as assessed by DNA measurements and ^{3}H TdR-labelling *in vitro* is strikingly lower.

The relative increase of lymphoid cells may be due to common hypocellularity or to elective decrease of erythropoietic cells as was often described in this condition. However this opinion could not be confirmed by the myelogram data, showing a normal E:G ratio in most of the cases. Additionally an increase of lymphocytes was not observed. Therefore, an absolute and independent increase of this cell type is suspected. In different experimental works concerning the haemopoietic stem cell activity of the bone marrow 0.1–0.8% colony-forming cells were found in several laboratory animals [1, 4, 14]. The cells presumed to be stem cells [5, 6, 13] show morphological characteristics similar to those of the lymphoid cells studied in the present investigation. However, the data obtained failed to give any conclusion about possible stem cell properties.

The results of the autoradiographic and cytophotometric investigation of the lymphoid cells point to a considerable proliferation activity in healthy persons. The ^{3}H TdR-labelling index of 44 ± 11% is somewhat higher than the comparable value in guinea pigs of 35% [15] and 38.5% [10].

The subclassification of the lymphoid cells into a basophilic and a pale group has presented evidence of a different proliferation activity. The minimal labelling and the high amount of diploid DNA values point to an out-of-cycle population (G_0). This hypothesis may be supported by the findings of ROSSE [12] who demonstrated the same DNA synthesis time (t_s) of 7.3 h and generation time (t_g) of 14 h in guinea pig pale and basophilic lymphoid cells. If t_s and t_g are indentical in both cell groups, an extremely long G_1 period can be excluded. On the whole, the different proliferation pattern confirms the initially used cytological cri-

teria for cell subclassification. The observation of some cells of 'intermediate' type points to a possible transformation of pale cells into basophilic cells. Accordingly, the pale lymphoid cells show some ^{3}H-TdR-labelling but only few tetraploid cells.

Comparatively, the proportion of lymphoid cells in S and G_2 in patients with chronic renal insufficiency is very low. This suggests a decreased proliferation activity and an elevated amount of cells in G_0. However, a prolonged period as seen in erythropoiesis in chronic renal anaemia [8] cannot be excluded.

The differences of the proliferation pattern of lymphoid cells in healthy persons and patients suffering from chronic renal anaemia suggest an involvement of this cell type in the pathophysiologic mechanism of this disease. The disturbances of bone marrow lymphoid cells in chronic renal insufficiency may be due to (a) direct toxic effects of metabolic products [2, 3], (b) alteration of the immunologic defense mechanisms, or (c) decreased erythropoietin levels seen in these conditions. Concerning the type of chronic renal disease (pyelonephritis, glomerulonephritis), no differences in lymphoid cell proliferation defects could be observed. For the evaluation of specific differences, the study of further cases will be necessary.

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ε-Amino-Caproic Acid in the Management of Acute Promyelocytic Leukaemia

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Key Words. ε-Amino-caproic acid Leukaemia Promyelocytic leukaemia

Abstract In three cases of acute promyelocytic leukaemia remission was induced with cytosine arabinoside, daunarubicin and vincristine. The coagulopathy was controlled by EACA without resorting to the use of heparin. All three survived more than 85 weeks.

Acute promyelocytic leukaemia (APML) has been recognised as an uncommon and distinct form of acute granulocytic leukaemia (AGL), on both morphological and clinical grounds. The predominant cell type contains either heavy azurophilic granulation or sheaves of needle-like structures similar in staining properties to Auer rods but showing distinct electron microscopic appearances [7] Most patients with this form of AGL have had a rapidly fatal course. The disease has been considered to respond poorly to chemotherapy [5 7] Most deaths, however have occurred at an early stage in the illness and have been due to massive haemorrhage, frequently intracerebral [3 5 7] This profound haemorrhagic disorder is the most striking clinical feature of the disease. Primary fibrinolysis has been suggested as the mechanism, at least in some patients [1 2, 6] Most current opinion, however favours intravascular coagulation as the underlying process, on the basis of post mortem and laboratory studies [3 5 7] It has therefore been recommended that heparin be used as treatment for this haemorrhage, together with massive doses of donor platelets [3 5] The mortality rate in this illness has, however not fallen dramatically with the introduction of these regimes in fact, BERNARD *et al.* [1] found that all five of their patients so treated, died.

Of a total of 43 patients with AGL presenting to us between October 1971 and June 1975 three had APML. All three achieved control of haemorrhage with prolonged survival, following treatment with chemotherapy and ε-amino-caproic acid (EACA)

Case Reports

Case 1. A 35-year-old Caucasian female presented in October 1971 with profuse bleeding from the sites of dental extraction, with purpura, ecchymoses and bleeding from venipuncture sites. Haemoglobin 9 g/dl, white cell count 5,000/µl platelet count 10,000/µl. Bone marrow aspirate contained over 95% of abnormal granulocytic cells, many of which had 'sheaves' of intracytoplasmic needle-like structures, as were described by SULTAN *et al.* [7]. No coagulation studies were performed. Cytosine arabinoside, daunorubicin and vincristine in approximately half of the usual dose [4] were sufficient to induce a complete remission. Haemorrhage was successfully controlled by EACA, 4 g intravenously 4-hourly for a period of 18 days. 8 units of platelets were transfused in the 1st week of treatment. The patient survived for 145 weeks.

Case 2. A 39-year-old Caucasian female presented in March 1973 with extensive purpura, ecchymoses, vaginal and retinal haemorrhages. Haemoglobin 8.5 g/dl, white cell count 10,500/µl; platelet count 16,000/µl. Bone marrow aspirate contained over 85% granulocytic precursors, with dense azurophilic cytoplasmic granulation. A diagnosis of APML was made. Plasma fibrinogen 100 mg/dl; prothrombin time 28 sec (control 16.2 sec); thrombin time 22 sec (control 12 sec); fibrin degradation products over 40 µg/ml. Specific chemotherapy administered was similar to that in patient No. 1. EACA, 4 g intravenously 4-hourly was given, bleeding ceased on the 4th day. 12 units of platelets were administered in the 1st week. Complete remission was achieved within 22 days; the patient still survives, 139 weeks after initial diagnosis.

Case 3. A 27-year-old Caucasian female presented in December 1972 with purpura, ecchymoses, haematuria, gum, retinal and venipuncture site bleeding. Haemoglobin 4.5 g/dl, white cell count 75,000/µl, platelet count 10,000/µl. Bone marrow aspirate contained over 95% of abnormal promyelocytes similar to those in case 2. Plasma fibrinogen 130 mg/dl; prothrombin time 21 sec (control 15.4 sec) thrombin time 18 sec (control 12 sec); fibrin degradation products over 80, but less than 160 µg/ml. Specific chemotherapy was similar to that used in cases 1 and 2. EACA, 4 g intravenously 4-hourly for 10 days, allowed control of haemorrhage. 19 units of platelets were administered in the 1st week. Complete remission was chieved in 25 days; the patient survived for 86 weeks.

Discussion

It was because of the poor results reported with the use of heparin in APML that we decided to use EACA in the first of these patients. The remarkable and prompt control of her haemorrhage, and her subsequent rapid achievement of a stable complete remission, encouraged us to use EACA in the two other patients. Equally good results were obtained. Requirements for transfused platelets in these three patients was below that recommended by GRALNICK and SULTAN [5]

The control of haemorrhage for a sufficient period of time to allow effective chemotherapy to be administered enabled a remarkable sensitivity of the disease to this therapy to become evident. Complete remission in these three patients followed the administration of only approximately half of the dose of cytotoxic agents usually required. Subsequent relapses in patients 2 and 3 were equally speedily controlled by low-dose chemotherapy and EACA. Furthermore, the survival of these three patients has been longer than the median survival in our series for patients attaining complete remission (72 weeks). In 1973 BERNARD *et al.* [1] also reported an unusually long survival in patients with APML who survive the initial bleeding episodes and are treated with daunorubicin.

We feel, therefore, that APML is a variant of AGL which has a potentially relatively good prognosis, which unfortunately has been obscured by the high initial haemorrhagic mortality. The use of heparin in this disease, notwithstanding the theoretical arguments in its favour, has not resulted in a dramatic fall in early deaths. The success of an anti-fibrinolytic agent in controlling haemorrhage in three unselected patients with APML encourages us to suggest that such agents may have a greater part to play in the treatment of this disease than hitherto recognised.

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Immunoglobulins in Idiopathic Thrombocytopenic Purpura in Childhood[1]

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Key Words. Complement in ITP · Immunoglobulins · ITP · Splenectomy

Abstract. Levels of immunoglobulins G, A and M were determined in 55 children with idiopathic thrombocytopenic purpura (ITP) (32 acute, 18 chronic and 5 recurrent). Mean IgG levels tended to be low in acute ITP and returned to normal with recovery. In contrast, chronic and recurrent ITP had persistently low IgG and IgA levels. C_3 complement levels in 10 cases of acute ITP were normal. Seven patients with chronic ITP had their spleens removed, and IgG and IgA levels tended to rise, while IgM levels decreased in 4 patients after the operation. Low IgA levels observed at the onset of purpura in a child might favor the diagnosis of chronic or recurrent ITP.

Idiopathic thrombocytopenic purpura (ITP) is a syndrome of unknown etiology characterized by a low number of platelets in the peripheral blood, abundant megakaryocytes in the bone marrow and a short platelet life span [1]. The severity and duration of ITP vary considerably and acute and chronic forms have been delineated. While the chronic form has an immunologic pathogenesis, the high incidence of antecedent viral infections supports the assumption that acute purpura in children is of infectious or postinfectious origin [2–4].

Experimental and clinical observations by McMillan *et al.* [5] suggest *that platelet destruction, at least in chronic ITP is associated with the presence of an antibody (immunoglobulin) probably synthesized by the spleen.* The same authors report that the immunoglobulin synthesis *in vitro* by human splenic tissue from patients with ITP is five times greater

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than that by control splenic tissue. The study of BALDINI in 1966 [6] suggests that the spleen might have a dual role in the pathogenesis of ITP the production of autoantibodies and the destruction of antibody-coated platelets. In the light of these reports, a study of the serum immunoglobulins in children with ITP is of interest.

Patients and Methods

The study comprised 55 cases of ITP 28 males and 27 females. Age distribution varied between 3 months and 12 years, with a peak of 2–4 years of age with no significant sex difference. 38 were newly diagnosed cases of ITP of which 6 became chronic. In addition, 12 children with already established chronic ITP and 5 with recurrent episodes of acute ITP were studied. (Those patients whose thrombopenia persisted for a period of more than 6 months were classified as 'chronic ITP'.)

Initial laboratory investigations included a hemogram, bone marrow examination and direct antiglobulin test. Serum immunoglobulin levels were estimated by the single radial immunodiffusion method of FAHEY and MCKELVEY [7].

In cases of persistent or recurrent thrombocytopenia the studies were repeated at intervals. Immunoglobulins were compared to values obtained from 130 apparently healthy children of the same age groups. These were children attending the well baby clinic or public schools; complete history and physical examination excluded infections in these controls.

Results

Figures 1–3 show the levels of serum immunoglobulin G A and M plotted according to age. Although there was considerable scatter the mean level of IgG in patients with acute ITP was significantly lower than normal ($p<0\ 02$) (table I). In 10 patients with low IgG complement (C_3) levels estimated by single radial immunodiffusion method [7] were normal (range 83.5–193 mg%) with a mean of $123\ 9 \pm 31\ 1$ compared to normal values of 123–167 mg% with a mean of 140.2 ± 20.9 With recovery low IgG levels returned to normal. The IgA and IgM did not differ significantly from normal.

In chronic ITP the mean values for both IgG and IgA were significantly lower than normal ($p<0\ 001$) (table I fig. 1 2) and remained so on repeated examinations. As a whole IgM levels were elevated ($p<0\ 001$) (table I fig. 3) Splenectomy was performed in 7 patients with chronic ITP and table II illustrates the changes in the immunoglobulin levels following this procedure. In all patients the IgA level increased,

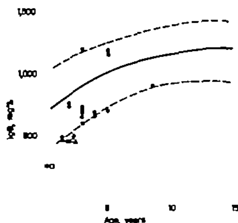


Fig. 1. IgG levels in patients with ITP. ● = Acute ITP; ▲ = incipient chronic ITP; ○ = chronic ITP; □ = recurrent ITP. Mean values (solid line) and SD (dotted lines) from 130 healthy children.

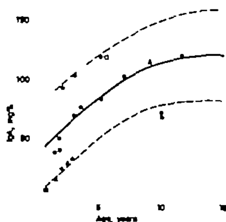


Fig. 2. IgA levels in patients with ITP. For legend see figure 1.

Table I Immunoglobulin levels in acute and chronic ITP in comparison with normal values

Group	Age, months mean ± SD	IgG mg % mean ± SD	IgA, mg % mean ± SD	IgM, mg % mean ± SD
Healthy children (n = 130)	63.34 ± 41.44	915.5 ± 235.5	102.00 ± 58.58	117.4 ± 41.14
Acute ITP (n = 32) P1 + 2	51.26 ± 32.45	788.7 ± 288.5 <0.02	81.09 ± 43.04 <0.23	120.4 ± 43.[illegible] <0.53
Chronic ITP (n = 18) P1 + 3	71.50 ± 37.2	721.2 ± 328.8 <0.001	65.60 ± 46.5 <0.001	153.0 ± 57.6 <0.001

Table II Immunoglobulin levels in 7 cases of chronic ITP before (a) and after (b) splenectomy

Case No.	Age	Sex	Duration		IgG mg %	IgA, mg %	IgM, mg
1	7 years	F	2 ½ years	a	950	35	240
				b	1,300	57	156
					(660–1 350)	(69–156)	(54–228)
[illegible]	23 months	F	1 year	a	450	54	9[illegible]
				b	693	263	54
					(436–1 178)	(31–98)	(52 [illegible]04)
3	2 ½ years	M	10 months	a	447	30	78
				b	493	93	100
					(557–1 100)	(31–98)	(52 204)
4	5 years	F	2 years	a	830	130	160
				b	1 067	216	147
					(680–1,270)	(42–116)	(55–214)
5	5 years	M	2 years	a	900	64	[illegible]00
				b	612	123	71
					(680–1,270)	(42–116)	(55–214)
6	9 years	F	1 year	a	1 050	73	[illegible]50
				b	1 158	111	240
					(660–1,350)	(69–156)	(69–278)
7	6 years	F	1 year	a	575	144	153
				b	817	169	218
					(660–1,350)	(69–156)	(54–228)

Values in parentheses are the normal ranges for the corresponding age.

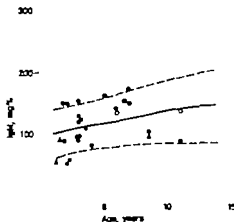


Fig. 3. IgM levels in patients with ITP. For legend see figure 1.

and in 6 the IgG level also increased, irrespective of the presplenectomy levels. IgM levels decreased in 4 patients, increased in 2 and showed no appreciable change in the seventh.

Four out of 5 patients having recurrent episodes of ITP showed low IgG levels and 3 low IgA, and these values were observed in successive recurrences and in between, so that the picture in recurrent purpura resembled that in chronic cases.

Discussion

The course of ITP in children is generally benign with spontaneous recovery. In the present series, 32 out of 38 children, recently diagnosed, recovered completely (84.2%) while the remaining 6 had chronic purpura. ITP in children usually occurs in close association with an apparent viral infection. The frequently observed time lag after such infection and the duration of thrombocytopenia is consistent with the assumption that circulating virus is not the sole etiologic factor. It has been suggested that a virus may associate with the platelet and produce its destruction by an interaction with antibody in a manner similar to certain drug-induced thrombocytopenia [8]. An antigen-antibody complex could be formed which attacks the platelets nonspecifically, the platelets here being the

passive target [9] Utilization of antibody might thus explain the lower range of IgG levels we found in the acute phase of ITP and the return to normal values with clinical and hematologic improvement. If such an antigen-antibody reaction is involved it does not appear to be C_3 dependent, as complement levels in the patients studied were within the normal range It is worth mentioning that there was no significant change in IgA or IgM levels.

By contrast, the chronic cases of ITP in our series had significantly lower mean IgA as well as IgG levels ($p<0.001$), whereas the IgM values increased in nearly half the cases (mean 153 ± 57 64 $p<0.001$). In the small series of recurrent purpura the same pattern was apparent. Despite the overlap of values, our data suggest that low IgA levels observed at the onset of purpura in a child might favor the diagnosis of chronic or recurrent ITP without any prognostic value in the individual patient. Chronic ITP in children resembles that seen in adults with general features of an immune disorder [10] Selective IgA deficiency has been reported to be associated with autoimmune diseases [11–14]

After splenectomy the levels of IgA showed a consistent rise in all 7 patients and those of IgG rose in 6. IgM levels showed no consistent pattern The seemingly paradoxical rise of IgG and IgA after splenectomy is in accord with the findings of SEITANIDIS *et al.* [15] in patients with thalassemia and has been attributed to lymphoreticular hyperplasia.

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The Effects of Late Thymectomy on the Immunosuppressive Action of Cortisone and Testosterone in Rats Immunized to Sheep Red Blood Cells

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Key Words. Cortisone Hemagglutinins Immunosuppression Lymphocytes Plaque- and rosette-forming cells Testosterone Thymectomy

Abstract. The immunological effects of cortisone and testosterone were studied in intact and lately thymectomized rats immunized to sheep red cells. The results showed that the effects of low doses of cortisone as well as those of high doses of testosterone (drop in the total number of rosette- and plaque-forming cells: RFC and PFC) were conditioned by the involution of the thymic cortex provoked by such hormonal doses, since these changes did not occur in thymectomized rats. The effects of high doses of cortisone were partly not influenced by thymectomy (drop in the RFC and PFC levels) but partly thymus-dependent (drop in the serum antibody titers). The latter were apparently related to advanced lesions of the thymic medulla.

The lymphocytic action of glucocorticoid hormones concerns mainly the thymus-dependent (T) lymphocytes [3 9–11] and that seems also to be the case of the immunosuppressive effects of these steroids [7]. Therefore, the thymic involution induced by the same hormones might be responsible for all these disturbances. However it has been argued that cortisone acts almost exclusively on the lymphocytes of the thymic cortex and does not injure notably the cells of the thymic medullary area, although these cells have been claimed to be the main contributors to the immune potential of the thymus [1 6].

Results obtained by comparing the immunological and hematological changes induced by low or high doses of cortisone in intact rats and in thymectomized rats, reported in the present paper suggest the hypothesis that the effects of low doses are due mainly to the drop in the number of cortisone-sensitive cortical thymocytes, whereas the effects of high doses

are conditioned either by the injury of the so-called cortisone-resistant lymphocytes of the thymic medulla or by a direct hormonal action on the peripheral immunocytes. Because of the thymolytic action of the androgens [2], and of some reports suggesting an immunosuppressive potential of testosterone [12, 14], the effects of this hormone were also investigated according to the same schedule as those of cortisone.

Methods and Materials

Two studies were performed on male adult rats of pathogen-free Sherman strain (CNRS, Orléans) fed with balanced commercial diet (CNRS). Each study included intact (I) rats and rats thymectomized (Tx) at 25 days of age. The absence of any thymic residue was verified after killing. I and T rats were littermates. At the age of 85 days the animals (weighing about 320 g) were immunized either with single intraperitoneal injection of 6×10^8 sheep red blood cells (SRBC) (study 1) or with two weekly subplantar injections of SRBC (3×10^8 into each pad) incorporated into Freund' adjuvant (study 2). The rats were killed by cardiac exsanguination one week after immunization(s).

I the study 1, low doses of hormone (1 mg/day cortisone acetate or 2 mg/day testosterone propionate) were given subcutaneously for 10 days from day 3 before immunization. In the study 2, the rats received much higher doses of hormones (10 mg/day cortisone; 7 mg/day testosterone) for 10 days starting on day 3 before the second immunization. In both studies, nontreated I and T rats were used as controls.

On the day of killing, the lymphocytes were enumerated in the caudal blood and the thymuses and spleens were weighed in the two studies, and also the popliteal lymph nodes in the second one. The thymuses were submitted to histological examination (hematoxylin and eosin). The immunological tests included counts of rosette-forming cells (RFC) [15] and plaque forming cells (PFC) [8] in the spleen (study 1) or in the popliteal lymph nodes (study 2). The counts were referred both to 10^3 and 10^6 viable lymphocytes respectively (the viability of the cells was evaluated by an erythrosin exclusion test [4]) and to the total cell population of the lymphoid organs. There were almost no 'spontaneous' RFC in nonimmunized rats [5]. Serum hemagglutinin titers were determined using microtitration plates [5]. Preparatory treatment of serums with 2-mercaptoethanol (2-ME) [5] permitted the separation of 2-ME-resistant immunoglobulins (IgG) from the 2 ME-sensitive Ig.

Results

Thymectomized Nontreated Rats

In the study 1 (single i.p. immunization), the Tx rats showed a significant decrease in the blood lymphocyte levels (–55.5%) and in the spleen

Table I Compared effects of cortisone and testosterone-propionate on the blood lympho-cyte levels and on the weight of the spleen (studies 1 and 2) and popliteal lymph nodes (study 2) in intact or thymectomized rats (means ± SEM)

Group	Study 1 blood lymphocytes/µl	Study 1 spleen mg	Study 2 blood lymphocytes/µl	Study 2 spleen mg	Study 2 popliteal lymph node, mg
Intact rats					
Nontreated (7)	13 690 ± 765	693 ± 22	11 160 ± 540	595 ± 23	91 ± 11
Cortisone-treated (8)	9.510 ± 500 (− 30 6)	561 ± 24 (− 19 1)	2 820 ± 255 (− 74.8) *	348 ± 15 (− 41.5)	25 ± 2 (− 72.5)**
Testosterone-treated (8)	10.340 ± 500 (− 24.5)	594 ± 7* (− 11 0)	1..430 ± 905 (+ 11 4)	555 ± 28 (− 6.7)	65.5 ± 4 (− 29 4)
Thymectomized rats					
Nontreated (8)	6.100 ± 200 (− 55.2)++	551 ± 20 (− 20.6)++	6.610 ± 370 (− 40.8)++	581 ± 24 (− 2.4)	56 ± 7 (− 38.5)*
Cortisone treated (9)	5.290 ± 310 (− 13.3)	496 ± 13 (− 9.9)	2.170 ± 240 (− 67.2)	328 ± 13 (− 43.5)*	17 ± 3 (− 69.9)*
Testosterone treated (8)	5 730 ± 350 (− 6.1)	479 ± 15 (− 13 0)	8.500 ± 1 065 (+ 28.5)	551 ± 15 (− 5.2)	62 ± 9 (+ 11.8)

The rats have been immunized against sheep red cells either by a single intraperitoneal injection (study 1) or by two weekly subplantar injections of the antigen (study 2). In parentheses numbers of rats and percent changes provoked either by thymectomy alone or by hormonal treatments. The treated intact or thymectomized rats were compared to nontreated rats of the same group.

and statistically different from nontreated group ($p < 0.05$ $< 0 01$ and < 0.001 respectively).

and ++ statistically different from nontreated intact group ($p < 0.01$ and < 0.001 respectively).

weight (–20 6%, $p < 0 001$) (table I) as well as in the number of RFC (–59.5%, $p < 0 05$) and PFC (–54 6%, $p < 0 01$) per whole spleen (table II) whereas there were no significant changes in the serum hemagglutinin titers (table III)

In the study 2 (two subplantar immunizations) significant lymphopenia (–40 8%, $p < 0 001$) and decrease in the weight of the popliteal lymph nodes (–38.5%, $p < 0 01$) were accompanied not only by a drop in RFC

Table II. Numbers of RFC and PFC in the spleen (study 1) or in the popliteal lymph nodes (study 2) in nontreated controls and in rats injected with cortisone or testosterone-propionate

Group	Study 1		Study	
	RFC, 10³	PFC	RFC, 10³	PFC
Intact rats				
Nontreated	1,596 ± 221	59,530 ± 11,290	553 ± 216	24,040 ± 6,860
Cortisone-treated	682 ± 148 (−57.0)*	24,560 ± 5,380 (−58.9)*	51 ± 14 (−90.7)*	3 620 ± 560 (−84.9)**
Testosterone-treated	2,330 ± 157 (+46.9)	50,440 ± 10,940 (−15.3)	146 ± 23 (−73.6)	8,790 ± 1,285 (−63.4)*
Thymectomized rats				
Nontreated	642 ± 178 (−59.5)+	27,020 ± 5,730 (−54.6)+	71 ± 49 (−87.2)+	6,360 ± 2,052 (−73.6)++
Cortisone-treated	401 ± 78 (−37.6)	21 060 ± 3,830 (−22.2)	12 ± 2 (−83.6)*	940 ± 339 (−85.2)**
Testosterone-treated	742 ± 247 (+15.7)	27 750 ± 4,220 (+2.7)	86 ± 35 (+21.5)	8,530 ± 3,105 (+34.1)

In parentheses percent differences between nontreated thymectomized and intact rats and between nontreated and treated animals in each group. The rats are the same as in table I.

p<0.05 p<0.01
p<0.05 ++ p<0.01

(−87.2%, p<0.05) and PFC (−73.6%, p<0.01) levels in these lymph nodes (which in this study replaced the spleen as the main organ concerned in the immune response) (table II) but also by a significant (p<0.05) although slight (−13.3%) fall in serum IgG hemagglutinin titers (table III). Thus the immunodepressive effect of late thymectomy was more pronounced in the secondary immune response (study 2) than in the primary one (study 1). Indeed, the thymus is mainly concerned with the regulation of the IgG production [13] which reaches much higher levels during the secondary response than after a single immunization (table III).

Cortisone-Treated Rats

Hematology. Low doses of cortisone (study 1) reduced slightly but significantly the weight of the thymus (−25%, 667 mg ± 28 → 499 mg ± 25

Table III Titers (±SEM) of total and 2 ME-resistant (IgG) serum hemagglutinins following treatment with low doses (study 1) or high doses (study 2) of cortisone or testosterone in intact and thymectomized rats immunized to sheep red cells

Group	Study 1		Study 2	
	total	IgG	total	IgG
Intact rats				
Nontreated	4.214±0.161	3.010±0.218	4 615±0.071	4 114±0.100
Cortisone-treated	3 951±0.105	2.860±0.099	4.281±0.084	3.445±0.167*
Testosterone-treated	3 838±0.094	2.747±0.192	4.386±0.145	3.863±0.121
Thymectomized rats				
Nontreated	3.875±0.120	2.709±0.161	4.214±0.131	3.569±0.153+
Cortisone-treated	3.645±0.170	2.542±0.225	4.269±0 124	3.424±0.148
Testosterone-treated	3 800±0.055	2.822±0.113	4.429±0.127	3.741±0.129

The animals are the same as in tables I and II Titers expressed as log 10 of geometric means of the highest serum dilutions provoking hemagglutination.

* Statistically different from nontreated group (p<0.01).

\+ Statistically different from nontreated intact group (p<0 05).

p<0 001) The histological lesions were limited to the cortex. On the contrary high doses (study 2) provoked a deep atrophy of this organ (-89%, 386 mg ±33 → 41 mg ±4 p<0 001) involving not only the cortex which disappeared almost completely but also the medullary area, which was considerably reduced and exhibited severe cytological lesions (mainly pycnosis and deformation of the nuclei)

Significant blood lymphopenia (-30% p<0 001) was induced by low doses of cortisone in I rats while the hormone was completely inefficient in Tx rats (p>0 05) which were already lymphopenic prior to the cortisone treatment. On the other hand the strong lymphopenia due to high doses dropped into the same relative level (as compared to the level of nontreated rats of each group) in I (-75%) and Tx rats (-67%) (table I).

Similarly the decrease in the weight of the spleen due to low doses of cortisone (study 1) was prevented by thymectomy (highly significant difference of -19% p<0 001 between nontreated and cortisone-treated rats in the I group no significant difference in the Tx group) whereas both I and Tx rats treated with high doses of cortisone (study 2) exhibited the

same weight drop of the popliteal lymph nodes and the spleen in the I group (–73 and 41.5%) respectively) and in the Tx group (–70 and –43.5%).

Immunology. In both studies, cortisone provoked a sharp drop in the total number of RFC and PFC per whole spleen or popliteal lymph node respectively (table II) but did not change significantly the concentration of these immunocytes in the two organs. Indeed, in the study 1 (single immunization) the number of RFC per 10^3 viable splenic cells was 3.18±0.45 and 2.14±0.58 in nontreated and treated I rats, and the number of PFC per 10^6 cells was 116±21 and 77±20 respectively. In the study 2 (two immunizations) the corresponding values were 10.00±2.85 and 11.36±2.88 per 10^3 for the RFC, 475±84 and 866±203 per 10^6 for the PFC in the popliteal lymph nodes. Obviously, the higher levels of both categories of immunocytes found in study 2 were due to the stronger immunization used in the latter.

With low doses of cortisone (study 1), the hormone-induced fall in RFC and PFC values per whole spleen was much more pronounced and statistically significant ($p<0.01$ and <0.05 respectively) in I rats (57 and 59% as compared to nontreated controls) than in Tx rats (nonsignificant decreases of 38 and 22% respectively). On the other hand, the mean drop in the RFC and PFC numbers in popliteal lymph nodes provoked by high doses of cortisone (study 2) was similar in the I group (–90 and –85%) and in the Tx group (–83 and –85%) (table II).

The hemagglutinin titers were not influenced by low doses of cortisone given during a primary immune response (study 1) but decreased with high doses during a secondary response (study 2, table III). The decrease was statistically significant only for the 2 ME-resistant IgG ($p<0.01$). By contrast to the levels of RFC and PFC, the drop in the IgG titers after high doses of cortisone was observed only in I rats but not in Tx rats which displayed reduced titers of these antibodies even in the absence of cortisone (table III). Similar data (not presented here) were found concerning the hemolysin titers.

Testosterone-Treated Rats

This hormone always provoked a highly significant drop ($p<0.001$) in the weight of the thymus (–70% in the study 1: 667 mg ± 28 → 203 mg ± 15; –87% in the study 2: 386 mg ± 33 → 48 mg ± 5) but even with very high doses (study 2) the thymic involution concerned almost exclusively the cortex while the medullary area remained relatively intact. There were

no significant changes in the levels of blood lymphocytes nor in the weight of the spleen and popliteal lymph nodes ($p>0.05$) (table I)

Only the highest doses of testosterone reduced significantly (-63%, $p<0.05$) the total number of PFC in the lymphoid organs studied (popliteal lymph nodes) of I rats. Such a reduction was not observed in testosterone treated Tx rats (table II) The titers of serum antibodies were never decreased after testosterone injections (table III).

Discussion

Cortisone. In the light of our experimental data, the hematological as well as immunological action of low doses of cortisone appear to be prevented by thymectomy and that is also the case of the decrease in serum IgG levels provoked by high doses of this hormone. These results suggest that the action of low doses is conditioned more or less completely by alterations of the thymic cortex, since only the latter is injured by such doses. This hypothesis has already been tested and confirmed in previous investigations [3] in which late thymectomy had almost completely prevented the transitory drop (maximal at 4 h) in the blood lymphocyte levels following a single intraperitoneal injection of a small dose of cortisone (1 mg/100 g body weight) in the rat.

There is no reason to believe that the blood lymphopenia as well as the fall in the spleen weight and in the number of splenic immunocytes induced by low doses of cortisone may be difficult to detect in animals already moderately lymphopenic due to thymectomy Indeed, the values found in the Tx rats were still far from having reached a maximal degree and a limit which did not permit any additional decrease. In actual fact, the constatation that adult thymectomy alone already determines a significant lymphopenia and a certain degree of immunodepression, does corroborate the hypothesis that the lympholytic and immunosuppressive action of low doses of cortisone is mediated mainly by the hormonally induced involution of the thymus, especially by that of the thymic cortex, the almost exclusive target of such doses of cortisone

The hypothesis above does not interfere with certain findings, according to which glucocorticoids seem to provoke also a redistribution of peripheral blood lymphocytes predominantly to the bone marrow [7 10], since such an effect should be considered only as additional as compared to the major thymolytic action of these hormones.

In contrast with the effect of low doses active mainly against the thymic cortex, the changes in the serum antibody titers, observed only with high doses of cortisone but also prevented by thymectomy were probably conditioned by the deep alterations of the thymic medulla provoked by such doses in addition to the destruction of the cortex.

On the other hand, the drop in RFC and PFC levels, the blood lymphopenia and the involution of the spleen and the lymph nodes, induced by the same intensive hormonal treatment, apparently did not depend on the status of the thymus, since these changes were not influenced by thymectomy Therefore they seemed to be due to a direct extrathymic noxious action of glucocorticoids on the peripheral T cells and perhaps also on B lymphocytes.

Testosterone Despite its strong thymolytic action, this hormone did not influence significantly the level of the blood lymphocytes nor the weight of the peripheral lymphoid organs. This surprising finding seems to indicate a relative conservation of the lymphopoietic potential of the thymus as well as that of the process of maturation and emigration of T lymphocytes from the thymus. Only very high doses of testosterone succeeded in provoking a drop in the total PFC levels in the lymph nodes. Being prevented by thymectomy this action appeared to be due to the hormone-induced injury of the thymic cortex as it was the case with low doses of cortisone. The relative integrity of the thymic medulla may explain why even very high doses of testosterone did not reduce the levels of circulating antibodies in our experiments.

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Erythropoietin Level and Thrombocytopoiesis in Mice

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Key Words. Erythropoietin · Megakaryocytes · Thrombocytopoiesis

Abstract. After administration of a single 1 IU dose of exogenous erythropoietin to mice or by stimulating endogenous erythropoietin production with androgen, the animals' thrombocytopoiesis, measured with selenomethionine incorporation, decreased. The suppression of endogenous erythropoietin level by hypertransfusion also caused a decrease of thrombocytopoiesis.

Certain changes in the erythrocyte system are associated with alterations of thrombocytopoiesis (posthemorrhagic thrombocytosis [9], thrombocytosis developing in anemia due to iron deficiency [2]). A certain relationship is supposed between the functions of these systems. However, at present it is commonly presumed that the two systems are separately regulated by special poietins (erythropoietin respectively thrombopoietin).

Our experiments aimed to answer the question, what kind of changes are produced in megakaryocytopoiesis by a change in erythropoietin level if either of exogenous intake, endogenous stimulation, or endogenous suppression are concerned.

Materials and Methods

The experiments were carried out on 3-month-old male mice of the CLFP strain weighing 30 g on average.

The ^{75}selenomethionine (Se-Met) incorporation method described by Penington [10] was applied to determine the rate of thrombocytopoiesis. Each mouse received 2 μC isotope intraperitoneally and we evaluated the 72-hour incorporation. Activity was measured with the Packard liquid scintillation automatic sample-changer apparatus.

Exogenous erythropoietin intake. Each mouse received 1 IU human erythropoietin isolated from urine, intraperitoneally in a single dose, 24, 48 or 72 h prior to the administration of the isotope. Erythropoietin was supplied to us by the WHO International Laboratory of Biological Standards, Mill Hill, London, for which we would like to express our thanks.

Endogenous erythropoietin stimulation. 6, 4 and 2 days before the administration of the isotope, 2.5 mg testosterone-propionate was given intramuscularly to each mouse, three times in all.

Endogenous erythropoietin suppression. Each mouse received i.p. 0.5 ml washed mouse erythrocyte mass, on two occasions, 6 and 3 days prior to the administration of the isotope. On the third day after administration of Se-Met the hematocrit value was between 70 and 78%.

Results

No change in thrombocytopoiesis occurred 24 and 48 h after the administration of exogenous erythropoietin but 72 h later it showed a visible decrease (table I). Similar effects were observed when endogenous erythropoietin was stimulated by repeated administration of testosterone-propionate. Compared to control animals thrombocytopoiesis of the treat-

Table I. Effect of 1 IU exogenous erythropoietin 1–3 days before the administration of ^{75}Se Met on the thrombocytopoiesis in mice

	72 h ^{75}Se-Met incorporation $\% \times 10^{-3} \pm 1$ SD	Number of mice
Control	11.7 ± [illegible].9	15
–24 h	10.0 ± 1.9	5
–48 h	9.9 ± 4.7	10
–72 h	6.1 ± 1.7	13

Table II. Effect of 3 × 2.5 mg testosterone-propionate 6–2 days before the administration of ^{75}Se-Met i.m. on the thrombocytopoiesis in mice

	72 h ^{75}Se-Met incorporation $\% \times 10^{-3} \pm 1$ SD	Number of mice
Control	9.4 ± 1.2	5
Testosterone-propionate	5.3 ± 2.2	10

Table III. Effect of erythrocyte hypertransfusion (hematocrit 70–78 % at the end of the experiment) on the thrombocytopoiesis in mice

	72 h ^{75}Se-Met incorporation % × 10^{-4} ± 1 SD	Number of mice
Control	9.5 ± 1.0	5
Hypertransfusion	7.7 ± 0.7	9

ed mice decreased (table II). Suppression of the endogenous erythropoietin level, induced by increasing the hematocrit value, similarly reduced thrombocytopoiesis (table III).

Discussion

Changes in erythropoiesis are frequently incidental to changes in thrombocytopoiesis. In patients with chronic hemolytic anemia, HIRSCH and DACIE [7] found an inverse relation between the hemoglobin level and the platelet count several months after splenectomy. CHOI and SIMONE [2] observed increased thrombocytopoiesis in rats with anemia due to iron deficiency. In cases of anemia produced by antierythrocyte antiserum, JACKSON *et al.* [8] observed thrombocytosis only if erythropoiesis was inhibited with actinomycin D. In the two latter cases thrombocytosis developed despite the inhibition of thrombocytopoiesis due to the presence of a presumably increased erythropoietin level.

In humans, SIRI *et al.* [12] observed a thrombocytosis-producing effect of hypoxia, whilst this was not stated by GROSS *et al.* [6]. EBBE and STOHLMAN [5] found that the erythropoietic urine extract of anemic persons was also thrombopoietic, but later they suggested [9] that the thrombopoietic effect could be correlated with thrombopenia present besides anemia, since anemic sheep plasma caused no increase in the platelet count. In cases of anoxia and polycythemia, DE GABRIELE and PENINGTON [3] detected no significant change in the platelet count of rats, while BIRKS *et al.* [1] observed thrombocytopenia in mice after hypoxia. A possible explanation for the above inconsistent data is that perhaps the erythropoietin was not only erythropoietically active, and results were evaluated upon the peripheral platelet count which could be affected by other factors, too.

SHREINER and LEVIN [11] observed the Se-Met incorporation increasing effect of human erythropoietin in rabbits. Contrary to this, we noticed decreased thrombocytopoiesis after a single erythropoietin injection or exogenous erythropoietin stimulation. Likewise, the endogenous suppression of the erythropoietin level had a reducing effect. According to the investigations of STOHLMAN *et al.* [13] a close connection exists between the stem cell pool and the committed stem cell pools (erythro- leuko- megakaryocyte systems).

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A Drug-Induced Haemolytic Anaemia due to Hb Torino (α43(CD1)Phe→Val). Second Finding in an Italian Family

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Key Words. Haemoglobinopathies · Hb Torino · Haemolytic anaemia · Inclusion body anaemia · Oxygen affinity · Unstable haemoglobins

Abstract. The unstable haemoglobin, Hb Torino (α43(CD1)Phe→Val), has been found for the second time in a family from the Treviso region of Italy. The haemoglobin has a slightly lower oxygen affinity than normal. In both cases, the abnormal haemoglobin is associated with inclusion body anaemia but the course of the disorder in the present case is much less severe than that previously reported. The oxygen affinity of the haemolysate has been measured and been found lower than normal.

We have had the opportunity to study a patient with inclusion body anaemia who has suffered several haemolytic crises since childhood, two of which followed the administration of sulphonamides. Laboratory tests showed the presence of an unstable haemoglobin which, on analysis, proved to be Hb Torino (α43(CD1)Phe→Val) first described in 1968 by Beretta *et al.* [1]. As did the family of the first case reported, the propositus and his family come from the Treviso region of Italy but they appear to be unrelated. This is, therefore, the second report of haemolytic anaemia due to Hb Torino but compared with the original case [20] the clinical and haematological status of our patient, out of crisis, is very much better.

Methods

Routine haematological studies were carried out by standard methods [10]. Paper electrophoresis was performed according to the method of Cradock-Watson

SHREINER and LEVIN [11] observed the Se-Met incorporation increasing effect of human erythropoietin in rabbits. Contrary to this, we noted decreased thrombocytopoiesis after a single erythropoietin injection or endogenous erythropoietin stimulation. Likewise the endogenous suppression of the erythropoietin level had a reducing effect. According to the investigations of STOHLMAN *et al* [13] a close connection exists between the stem cell pool and the committed stem cell pools (erythro- leuko- megakaryocyte systems)

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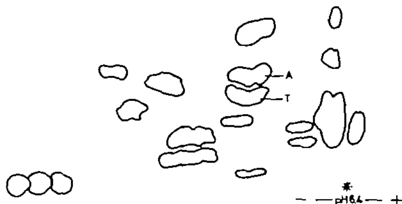


Fig 1 Fingerprint of the soluble tryptic peptides of aminoethylated α-chain isolated from the isopropanol precipitate prepared from fresh haemolysate of the propositus' red cells. Electrophoresis at pH 6.4, 55 V/cm for 1 h. Ascending chromatography in pyridine-isoamyl alcohol-water (6:6:7 by volume) for 18 h. * = Point of application. A = αTpVI T = TorinoTpVI

Family History

The parents, brother and sister of the propositus are all in good health. There is no history of haemolytic crises but the haemolysate of the father's red cells gave a strongly positive heat stability test, as did the haemolysate of the propositus' son. Therefore it can be concluded that three members of the family over three generations, have Hb Torino.

Structural and Oxygen Affinity Studies

Identification of the Abnormal Haemoglobin

Fingerprints of the aminoethylated α- and β-chains isolated from globin prepared from the crude haemolysate revealed no abnormal peptides in either chain. The aminoethylated α-chain prepared from the 10 min isopropanol precipitate, however had an additional peptide which migrated chromatographically on the fingerprint directly below αTpVI (fig. 1). The amino acid composition of this peptide corresponded to αTpVI with the exception that one of the two phenylalanine residues (α43 or α46) (table II) had been replaced by a residue of valine. The site of the substitution was determined by performing two successive steps of Edman degradation on the isolated abnormal TpVI and dansylating the new N-terminal amino acid exposed. After hydrolys-

SON *et al.* [7]. Hb A_2 was quantitated by cellulose acetate electrophoresis [17] and the proportion of HbF obtained by the method of SINGER *et al.* [22]. Isopropanol stability tests were performed as described by CARRELL and KAY [4] and heat instability tests according to DACIE *et al.* [8]. Assays for methaemoglobin [17], red cell G6PD levels [14], and GSH levels [7] were performed.

Globin samples were prepared [23] from the crude haemolysate and the precipitate which formed after 10 min incubation of the haemolysate with isopropanol at 37 °C. α and β-chains were isolated by chromatography of the globin samples on carboxymethyl cellulose (Whatman CMC23) [6] and subsequently aminoethylated [16]. Digestion of the aminoethylated globin chains with trypsin, fingerprinting of the tryptic peptides and the determinations of amino acid compositions of peptides were carried out as previously described [5]. Dansyl Edman degradation was performed on isolated tryptic peptides according to the method of GRAY [14]. Oxygen affinity measurements were made by the continuous recording method of IMAI *et al.* [15]. The measurements were made on haemolysates in 0.1 M potassium phosphate buffer at 20 °C.

Case Report

The propositus, born in 1947, reported icterus at 6 and 16 years of age. In 1966, during an episode of acute tonsillitis, he was treated with sulphonamides. A few days later, jaundice, pallor and asthenia were observed. At this time both the haemoglobin level and erythrocyte count fell but the level of reticulocytes increased and erythroblasts appeared in the peripheral blood. On this occasion, the diagnosis was acquired haemolytic anaemia (or G-6PD deficiency?). In September 1971, during another episode of tonsillitis, sulphonamides were once again prescribed. Jaundice and darkening of the urine were reported some days later. He was admitted to Galliera Hospital where he was first seen by us and extensive tests were carried out. Since this crisis the patient appears to have been in good health.

Laboratory Investigations

The haematological indices recorded between 1962 and 1975 are summarized in table I. In 1971, the following results were obtained: serum bilirubin 1.4 mg% (direct reacting fraction 0.6 mg%); direct and indirect Coombs test negative; serum iron normal; saline osmotic fragility decreased; methaemoglobin on fresh blood 0.01%, and after 48 h incubation at 37 °C 0.8%. Direct staining with brilliant cresyl blue revealed a very few Heinz bodies in the red cells after 24 h at 37 °C. After Heinz body induction *in vitro*, 15% of red cells contained five or more inclusions (control only 2%). The heat stability test and isopropanol stability tests were both positive after 10 min incubation at 37 °C. Electrophoresis of the haemolysate revealed no abnormal haemoglobin fraction and both HbA_2 and HbF levels were normal. Red cell G-6PD and GSH levels were also normal.

When the patient was further examined in 1974 and 1975, although he appeared to be in good health, haematological investigations revealed a slightly reduced red cell count, and haemoglobin level with an increased reticulocyte count.

Table II Amino acid sequence of TpVI from human haemoglobin

	Sequential No.															
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Helical notation	C6	7	CD1	2	3	4	5	7	8	D6	7	E1	2	3	4	5
Sequence Hb A	Thr	Tyr	PHE	Pro	His	Phe	Asp	Leu	Ser	His	Gly	Ser	Ala	Gln	Val	Lys
Hb Torino	Thr	Tyr	VAL	Pro	His	Phe	Asp	Leu	Ser	His	Gly	Ser	Ala	Gln	Val	Lys

Table I Haematological data obtained from the propositus

	1962	1966	1971	1974	[illegible]
Hb, g/100 ml	11 4	10.4	12		13
RBC × 10 /µl	4	3 15	4.1		4.2
PCV		29	38		41
Reticulocytes,		5	5	4	3.4
White cells/µl	6,900	11 000	6,900		7,[illegible]
Blood film					
Anisocytosis		+ +		+ +	+ +
Poikilocytosis		+ + +			+
Distorted spherocytes		+		+	+
Erythroblasts		+			—

is of the dansylated peptide with 6 N HCl for 16 h at 108 °C, the dansyl amino acid released was identified as dansyl valine by chromatography on polyamide thin layer plates. The unstable haemoglobin is therefore Hb Torino α43(CD1)Phe→Val which was first described by BERETTA *et al* [1] and PRATO *et al* [21] Since the abnormal αTpVI was below the limit of detection by the standard fingerprinting technique using purified α-chain from hemolysates an estimate of the proportion of the abnormal haemoglobin in peripheral blood was impossible. However in view of the fact that Hb Torino amounted to less than 50% of the isopropanol precipitate (fig. 1 table III) its proportion is most probably small, possibly of the order of 8% or less as reported by BERETTA *et al* [1]

Oxygen Affinity of Hb Torino

The haemolysate containing Hb Torino had a p50 value about 13% higher than that for the control haemolysate (table IV) This indicates that Hb Torino has a somewhat lower oxygen affinity than Hb A which confirms a previous suggestion by GALLO *et al* [13]

Discussion

Phenylalanine CD1 is an invariant residue in all known haemoglobins and myoglobins [11] making close contact with the haem group [19] In addition to Hb Torino two other abnormal haemoglobins are known

structure, the phenylalanine side chain stabilises the haem group in the more inclined position it adopts in the tertiary oxy structure. In Hb Torino, the substitution of this important residue by the smaller valine residue results in a weaker bonding between haem and globin and produces a gap at the surface of the haem pocket. Furthermore, the valine can also make contact with CD4 phenylalanine thereby disturbing the conformation of the CD segment. These conformational changes cause the instability of the molecule. The altered oxygen affinity is most probably the result of the inability of valine to substitute for the larger phenylalanine in stabilising the haem group at the angle of inclination required in the tertiary oxy structure thus producing a shift in the equilibrium between the two allosteric forms in favour of the deoxy conformation.

The haemolytic crises in the propositus were not so severe as those reported earlier in the first case, by PRATO *et al.* [21]. They were self correcting contrasting with the transfusion need and ultimate splenectomy required by the original case. The onset of the last two crises in the propositus was clearly associated with the administration of sulpha drugs and there is no evidence of spontaneous crises. The first case, on the other hand, had several spontaneous crises and one triggered by sulphonamides. Out of crises, the propositus experienced good health although the haematological evidence during these periods suggests a compensated spontaneous haemolysis. The first case, on the other hand, suffered from persistent pallor and anaemia. Indeed PRATO *et al.* consider that the anaemia has persisted since birth on account of the presence of certain 'mongoloid' features. The difference in the severity of the two cases is difficult to explain since the available evidence implicates the abnormal haemoglobin as the only biochemical defect. Perhaps the case of PRATO *et al.* had an additional, as yet undetected, metabolic defect and one is tempted to suggest a possible interaction with α-thalassaemia.

While the two families are apparently unrelated, it is unlikely that the abnormal α-chain arose independently in both as a spontaneous new mutation since both come from such a small geographical area. The most logical explanation is that they have a common ancestral carrier of the abnormal gene.

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Table III Amino acid composition of αTpVI from Hb Torino

Amino acid	nmol		Molar ratio	
	α^{Torino}TpVI	α TpVI	α^{Torino}TpVI	α^{A}TpVI
Asp	12.3	17 7	0.98	1.06
Thr	10.5	14 0	0.84	0 84
Ser	22.9	31.3	1.83	1 87
Glu	13..	18 6	1 10	1.11
Pro	12.5	17 1	1 00	1 02
Gly	11 9	18.2	0.95	1 09
Ala	1..5	17 1	1 00	1.02
Val	4.3	17.2	1 94	1 03
Leu	12.8	17 8	1 00	1.07
Tyr	1..8	16.6	1 00	1.00
Phe	12.9	33.3	1 00	1.99
His	4 6	32.6	1 97	1.96
Lys	1..5	16.7	1 00	1.00

Table IV Oxygen affinity data for normal and abnormal haemolysate containing Hb Torino

Haemolysate	pH	p50 mm Hg	log p50
Normal	6.450	11 0	1.04
Torino	6.410	13 5	1 13
Normal	7.090	7 8	0 89
Torino	7 050	9 3	0.97
Normal	7 400	5 8	0.76
Torino	7 390	6.5	0.81

involving the homologous position in the β-chain β42(CD1)Phe Hb Hammersmith Phe→Ser [9] and Hb Bucaresti Phe→Leu [3] These three haemoglobins share two properties instability resulting in a haemolytic anaemia and lowered oxygen affinity

The molecular basis of the instability and altered oxygen affinities of these haemoglobins has been extensively discussed elsewhere [18, 20] In brief the bulky hydrophobic phenylalanine residue CD1 forms part of the haem pocket The benzene ring makes Van der Vaal contacts with the neighbouring porphyrin ring of the haem group In the conformational changes occurring during the transition from tertiary deoxy to oxy

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Materials and Methods

Routine haematological studies, cellulose-acetate electrophoresis and starch-gel electrophoresis of the haemolysates, and the karyotype, were performed by standard methods. Electrophoresis of the globin chains was carried out as described by Komarmy and Barnes [17], the thermal stability kinetic pattern as described by Wajcman *et al.* [24], the isopropanol test as described by Carrell and Kay [6], the glycerol lysis time as described by Gottfried and Robertson [15]. Haemolysates were fractionated using column chromatography on DEAE Sephadex according to the method of Huisman and Dozy [16], and globin was prepared by acid-acetone precipitation at 0 °C (washing the globin 3 times in acetone at 0 °C) [1]. The α- and the non-α polypeptide chains were separated and then aminoethylated as described by Clegg *et al.* [8]. The aminoethylated chains were digested with trypsin and the resulting soluble peptides were finger-printed using high-voltage electrophoresis at pH 6.4 and ascending chromatography [20]. The diagnostic finger-prints were stained with ninhydrin (0.2% in acetone) and reagents specific for methionine, histidine, arginine, tyrosine and tryptophan [22]. The preparative finger-prints were developed with ninhydrin (0.02% in acetone) and peptides were eluted in constantly boiling 6 N HCl, hydrolysed in sealed capillary tubes at 110 °C for 18 h, dried in vacuo and subsequently analysed on an automatic amino acid analyser.

Case Report

The patient was a 44-year-old white woman born in Tarifa, Cádiz, Spain, with two children aged 10 and 13, and no history of abortions. She had an attack of jaundice at the age of 8. Otherwise she is in good health, although she tires easily and shows pallor and some diffuse biliar dyspepsia. A cholecystogram suggested the presence of gallstones. No organomegalia, no coliuria, no hyperpigmentation of faeces, no pruritus were found. Her gynaecological story was normal and there were no abnormal blood losses. The conclusion from the basic haematological studies was: sideropenic hypochromic microcytic anaemia (haemoglobin 8.5 g%) with the presence of a pathological haemoglobin fraction (10% as quantitated from eluates on cellulose-acetate electrophoresis). The electrophoresis of the chains and the heat and isopropanol stability tests were normal. No Heinz bodies were found. The usual haemolysis tests were normal, except for the osmotic fragility tests and the glycerol lysis time which both showed an abnormal thalassaemia-like pattern. The karyotype was normal. The parents of the patient died at the age of 62 and 76 years with no history of haemolytic anaemia. The clinical and haematological studies of the 2 children were normal. A study of the rest of the family was not possible because some members refused to cooperate. Only 2 sisters were screened, one of them showed the same abnormal trait. Radiological and endoscopic gastroduodenal explorations of the patient showed no organic lesion and the benzidine test of the faeces was negative. A oral iron therapy was started with good absorption and response. On the 40th day the haemoglobin level was 11.7 g%. After 17 months, the patient remained haematologically stabilised, and experienced

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Haemoglobin Lepore Boston in a Spanish Family

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Key Words Haemoglobin Lepore Boston Haemoglobinopathies Thalassaemia

Abstract A case of haemoglobin Lepore is reported in a Spanish patient who has a sideropenic anaemia and possibly a thalassaemia trait. The carrier's clinical and haematological data are described The structural analysis of the haemoglobin showed it to be haemoglobin Lepore Boston.

Haemoglobin Lepore Boston was the first Lepore variant to be reported [12] and it has now been found in different populations in Italy [21] Rumania [19] Yugoslavia [9] Greece [11] and Cyprus [4] In 1961 one of us (H L.) with Dr F Barros of the Hospital de Santa Maria Lisboa, studied a Portuguese family in which both haemoglobins S and Lepore occurred with haemoglobin A in some members of the family and haemoglobin Lepore with haemoglobin S in one of them in whom it gave rise to a modified sickle cell anaemia The haemoglobin Lepore was of the Boston type Though communicated at a Congress, this family was not published in detail

Structural analyses [2, 3 18] have shown the two non-α chains of the variant to have a sequence identical to the γ-chain from residues 1–87 and to that of the β-chain from residues 116–146 thus showing that it is the product of an unequal crossing-over between the γ- and the β-structural loci. A further finding of this haemoglobin variant this time in Spain forms the basis of this report.



Fig 1 Haemoglobin Lepore Boston: finger-print of the soluble tryptic peptides of the aminoethylated non-α(δβ) chain.

XIIb (in the δ-chain, this peptide is split further to give two peptides δTp XIIb, and δTp XIIb, which remain closer to the origin). This indicated that the variant was a Lepore haemoglobin All the peptides which differ in amino acid composition between the δ- and β-chains were then analysed. Table II shows that peptides Tp II Tp III, Tp V and Tp X–XI had a δ-like composition, whereas Tp XIIb and Tp XIIIa gave a β-like analysis. This variant was therefore haemoglobin Lepore Boston with the δ- β-cross-over between residues 87 and 116.

Discussion

The clinical and haematological findings of the patient suggested a nutritional anaemia, whereas the indices according to ENGLAND and FRASER [10] the osmotic fragility patterns and some clinical data indicated a β-thalassaemia trait. It appears that the glycerol lysis time does not distinguish between iron deficiency microcytic anaemia and thalassaemia trait [13 15] While the structural haemoglobin analysis was carried out, a therapeutic trial was attempted, with success, and the patient became haematologically equilibrated. In the fairly wide Mediterranean occurrence of haemoglobin Lepore, Spanish ancestry of the patients has sometimes been suggested [5 23] Two possible cases have been found

Table I Haematological data of the patient

	Months 0	1	2.5	11	17
Haemoglobin g%	8.5	9	11 7	12.1	13
Haematocrit, %	30	30	37	35	33
RBC × 10⁶/μl	4 92		5.57	5 1	5.0
Reticulocytes × 10³/μl	68	128	89	35	113
MCV fl	60	60	66	69	66
MCHC, %	28	30	31	34	34
Osmotic fragility	decr			decr	decr
Glycerol lysis test, sec	85			86	
WBC × 10³/μl	5.0	6.4	5.0	5.0	4.7
ESR mm/1 h	14		14	5	[illegible]
Serum iron μg %	21	280	105	—	110
TIBC, μg	363	344	234	360	315
Sat. transferrin %	5	81	44	6	35
Serum haptoglobin mg %	1.5			1.5	125
Serum bilirubin mg	0.6			1 4	1.0
Direct fraction mg%	0.4			0 8	0.7
Serum haemoglobin, mg%	0 9				
Serum vitamin B_{12}, pg/ml				690	
Serum folate, ng/ml				14	17
Red cell folate ng/ml				352	513
Direct antiglobulin test	neg.			neg	
Fetal red blood cells, %	0			10	5
Alkali-resistant haemoglobin %	0.3			0.55	2.6
Haemoglobin electrophoresis					
Haemoglobin A_1 %	86.1				89.9
Haemoglobin A_2 %	3 6				1.5
Pathol. slow fraction %	10.3				8.5

only a discrete fatigue when doing housework. Table I shows the haematological data of the patient.

Results

Figure 1 shows the finger-print of the soluble tryptic peptides of the aminoethylated non-α chain (that of the aminoethylated α-chain showed no differences from normal α-chain). Tp III was in the position of δTp III and not βTp III and also a peptide appeared in the position of βTp

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Table II Haemoglobin Lepore Boston amino acid composition (molar ratios) of the tryptic peptides which differ in structure between the γ- and β-chains (A) and expected amino acid compositions of the tryptic peptides of the γ-chain (B) and β-chain (C)

Amino acid	Tp II A	B	C	Tp III A	B	C	Tp V A	B	C	Tp X–XI A	B	C	Tp XIIb A	B	C	Tp XIIIa A	B	C
Asp	1.0.	1	0	1.80	2	2	2.90	3	3	3.07	3	3		1	0			
Thr	0.81	1	1					0	1	1.01	1	2				0.87	1	1
Ser		0	1				2.51	3	2	1.70		1						
Glu				1.07	1	–	1.11	1	1	3.05	3					1.70	3	
Pro							1.53	2	2	0.93	1	1				2.20	1	2
Gly	1.01	1	1	3.00	3	3	2.22	2	2	0.81	1	1	1.08	1	1			
Ala	2.17	2	2	2.12	2	1	1.17	1	1		0	1	1.08	1	1	1.93	2	2
Val	1.1	1	1	3.16	3	3	1.18	1	1	1.11	1	1	0.96	1	1	1.14	0	1
Met							0.84	1	1								1	4
Leu	0.99	1	1	0.89	1	1	1.05	1	1	3.17	3	3	1.09	1	1			
Tyr																0.60	1	1
Phe							2.89	3	3	2.03	2	–	1.02	1	1	1.07	1	1
His										2.08	2		1.95	0	2			
Lys	0.88	1	1				0.97	1	1	1.47	1	1	0.82	1	1			
as-Cys[1]											1	1						
Arg				0.96	1	1				1.04	1	1		1	0			
+Trp[2]		1	1															
Yield, (nmol/residue)	17.0			28.1			22.3			26.7			20.0			4.1		

[1] as-Cys is mostly destroyed during acid hydrolysis and is found in the same place as lysine.

[2] Peptide fluoresced under UV light and stained with Ehrlich reagent.

in Spain but biochemical studies of the haemolysates were not carried out [7]. Our case confirms the existence of haemoglobin Lepore Boston in Spain.

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β Thalassaemia Haemoglobin S and Hereditary Elliptocytosis in a Zairian Family

Ischaemic Costal Necroses in Child ith Sickle Cell β^{+} Thalassaemia

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Key Words. Costal necrosis Elliptocytosis Haemoglobin S Sickle cell β^{+}-thalassaemia Zaire

Abstract In family of Low Zaire, child with sickle cell β^{+}-thalassaemia disease, aged 7 presented 33.6% haemoglobin A. The clinical evolution was benign, except for the occurrence of an extensive bilateral ischemic necrosis of the ribs. This is the third case of sickle cell thalassaemia with the haemoglobin phenotype SFA reported in Zairians, whereas the SF phenotype has repeatedly been found. β^{+}-thalassaemia seems therefore much rarer in Zairians than β^{0}-thalassaemia. Besides the thalassaemia trait and the sickle cell trait, hereditary elliptocytosis was also segregating in this family: double heterozygotes for Hb S and elliptocytosis or for β-thalassaemia and elliptocytosis did not show any definite sign of genetic or clinical interaction between both traits.

Both sickle cell trait and β-thalassaemia are present in Zairian populations they differ very much in incidence, the former being much more frequent than the latter Still relatively many cases of heterozygous state for both traits, i.e. sickle cell β-thalassaemia, were reported. The majority of them are characterized by an apparently complete suppression of the β-chain synthesis, so that no haemoglobin A (Hb A) is detected at electrophoresis: this condition is termed sickle cell β -thalassaemia. Other affected individuals present detectable Hb A, but in lower proportion than the sickle cell trait carriers their β-thalassaemia gene does not suppress but only reduces the β-chain synthesis, this form, the so-called sickle cell β^{+}-thalassaemia, seems to be rarer in Zairians than the β -form, since only

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Key Words. Costal necrosis · Elliptocytosis · Haemoglobin S · Sickle cell β^+-thalassaemia · Zaire

Abstract. In family of Low-Zaire, child with sickle cell β-thalassaemia disease, aged 7, presented 33.6% haemoglobin A. The clinical evolution was benign, except for the occurrence of an extensive bilateral ischemic necrosis of the ribs. This is the third case of sickle cell thalassaemia with the haemoglobin phenotype SFA reported in Zairians, whereas the SF phenotype has repeatedly been found. β^+-thalassaemia seems therefore much rarer in Zairians than β^0-thalassaemia. Besides the thalassaemia trait and the sickle cell trait, hereditary elliptocytosis was also segregating in this family: double heterozygotes for Hb S and elliptocytosis or for β-thalassaemia and elliptocytosis did not show any definite sign of genetic or clinical interaction between both traits.

Both sickle cell trait and β-thalassaemia are present in Zairian populations, they differ very much in incidence, the former being much more frequent than the latter. Still relatively many cases of heterozygous state for both traits, i.e. sickle cell β-thalassaemia, were reported. The majority of them are characterized by an apparently complete suppression of the β-chain synthesis, so that no haemoglobin A (Hb A) is detected at electrophoresis, this condition is termed sickle cell β^0-thalassaemia. Other affected individuals present detectable Hb A, but in lower proportion than the sickle cell trait carriers: their β-thalassaemia gene does not suppress but only reduces the β-chain synthesis: this form, the so-called sickle cell β^+ thalassaemia, seems to be rarer in Zairians than the β^0-form, since only

two adult cases were reported up to now [8, 32]. We report here on a family with a new case in a child aged 7. In this family hereditary elliptocytosis was also segregating so that carriers of Hb S, of β-thalassaemia and of elliptocytosis were found, two of these traits combined in some members, so that heterozygotes for β thalassaemia and Hb S, for elliptocytosis and Hb S and for elliptocytosis and β-thalassaemia were found.

Methods

The current haematological data were obtained by standard techniques. The *in vitro* sickling tests were performed by the sodium metabisulphite method; haemoglobin solubilities were estimated according to ITANO [12]. Haemoglobin electrophoreses were made in starch-gel with a Tris-EDTA-borate buffer system, pH 8.5 and in agar-gel with an acid buffer [17]. Fetal haemoglobin was estimated by alkali denaturation according to BETKE *et al.* [5], but when the results exceeded 3%, the 1-min method of SINGER *et al.* [25] was applied; the distribution of fetal haemoglobin amongst the red cells was examined by a modification of the Kleihauer's elution test [13]. Hb A_2 was estimated photometrically after separation by preparative starch-block electrophoresis by a technique which allows to separate completely Hb A_2 from Hb S [30].

Case Report

The propositus (II, 8 in table I), born on May 7th, 1967, was admitted on July 25th, 1974 in the Paediatric Department of the University Hospital at Kinshasa (file No. 90,818); he had never been hospitalized before. He presented marked dyspnoea and hectic pyrexia with maxima at 39.5 °C. Examination showed a height (113 cm) and a weight (17.5 kg) slightly too low for his age, bilateral dullness at the inferior part of the thorax, and acute pain at palpation of the middle and lower ribs; splenomegaly (1 cm) was noted but no icterus. At admission, radiographic examination of the chest showed a bilateral pleuritis and round opacity at the right hile corresponding apparently to an adenopathy; 5 days later diffuse opacifications with marked periosteal reactions and bone thickening were noted at the 3rd rib on the right and at the 3rd and 4th ones on the left. The intradermoreaction with 10 U tuberculin was negative; several haemocultures and the serological tests for salmonelloses and for brucelloses gave also negative results. Serum proteins amounted to 7.8 g/dl and γ-globulins to 2.85 g/dl. Ascaris and Trichuris eggs were found in the stools. Haematological examination showed a hypochromic normocytic anaemia (table I); serum bilirubin (0.6 mg/dl), serum iron (67 µg/dl), iron binding capacity (270 µg/dl) and transferrin saturation (4.8%) were normal. Osmotic fragility was strongly decreased, since 50% haemolysis was at 0.25 g NaCl/dl and total haemolysis at 0.10. G-6-PD activity was normal. The blood smears showed a few sickle cells, patent hypochromia and strong anisocytosis and poikilocytosis, some target cells

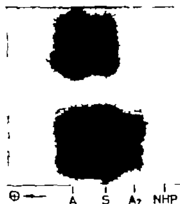


Fig. 1 Haemoglobin starch-gel electrophoresis, pH 8.5 Above: sickle cell trait carrier (Hb A > Hb S). Below: propositus with sickle cell β^+-thalassaemia (Hb S > Hb A, increased Hb A_2 and Hb F). NHP = Non-haem. protein.

schistocytes, spherocytes and red cells with basophilic stippling were noted. Sedimentation rate 79 mm/h. Leucocytes 13,200/µl of which 45% were neutrophils.

The sickling test was positive. Haemoglobin electrophoresis showed Hb S + Hb A + Hb A_2 pattern (fig 1); the relative amounts of the different fractions were Hb S, 57.0%, Hb A, 33.6%; Hb A_2, 5.0% and Hb F 4.2% The acid elution test showed strongly heterogeneous distribution of Hb F amongst the red cells

These findings entailed the diagnosis of sickle cells β-thalassaemia, which was confirmed by the family study and by the antecedents: the child indeed was never transfused; therefore the Hb A fraction was well his own. After fortnight treatment with antibiotics (penicillin-colimycin, then tetracycline), antipyretics and promethazine, the fever ceased abruptly The evolution of the radiographic abnormalities of the chest was as follows: 12 days after the admission the periosteal reaction had markedly progressed and affected the 3rd to 6th ribs on the right and the 3rd, 4th, 5th, 6th and 9th ribs on the left several foci of necrosis had appeared on both sides. 15 days later the bone necrosis was particularly obvious at the 5th and 6th right ribs in form of two oval lacunae (fig 2), but the pleural reaction was regressing. Finally on September 24th, 2 months after the onset, the periosteal reactions had disappeared and the bone structure of the affected ribs was normalizing; in particular the lacunar aspect of the necrotic foci was much less conspicuous, the hilar adenopathy had partially regressed.

During the whole hospitalization, the child remained anaemic, the haemoglobin levels varying between 7.2 and 10.8 g/dl. He was discharged 12 weeks after his admission, with prescription of regular folic acid administration. H attended the out patient clinic and was found in good condition 6 months later the splenomegaly had disappeared, but the hypochromic anaemia was still present, the haemoglobin level and MCHC being 8.9 and 28.7 g/dl, respectively

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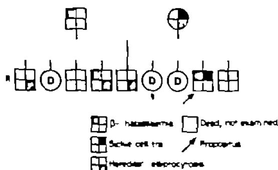


Fig 3 Pedigree of the family 1 case II, 5 paternity is excluded

The *eldest* / *the sons* (II, 1), aged 25 was healthy except that the blood smears showed about 90% elliptic-shaped cells; therefore, he had only inherited elliptocytosis from his mother apparently without ny harmful efect.

The *second son* (II, 3) 22 years old, had no sign of thalassaemia or of elliptocytosis and had not inherited Hb S from his mother A few throphozoites of *P falciparum* were found in the blood smears and some eggs of *Necator americanus* in the stools; these findings explain the anisocytosis and the slightly decreased MCHC (table I)

The *third son* (II, 4), aged 20, presented with markedly hypochromic anaemia Hb A_2 was markedly increased, serum iron was normal (table I) β-thalassaemia was further substantiated by lowered osmotic fragility (50 and 100% haemolysis at 0 35 and 0 10 g NaCl/dl respectively) nd by conspicuous characteristic abnormalities of red cells: anisomicrocytosis, numerous poikilocytes, schistocytes, pear-shaped erythrocytes, spherocytes and numerous target cells. Furthermore, about 50% of the red cells were definitely lliptic, some of them were very long and strongly coloured with parallel sides; these 'rod forms' or 'bacteriocytes' are characteristic of hereditary elliptocytosis and are not found in the cases of anaemia in which symptomatic elliptocytes occur [38]. This patient therefor combined β-thalassaemia and hereditary elliptocytosis, each trait being inherited from one parent, he was never hospitalized, his spleen was not palpable and no other clinical abnormality w found, his eight, 58.5 kg, was normal for his height, 169 cm, consequently in this subject the association of both traits gave no evidence of clinical nteraction.

The *fourth son* (II, 5), 17 years old, was slightly anaemic without haemoglobin abnormality (table I). *Necator* eggs were found in his stools Blood smears showed about 90% of elliptocytes, but no definite rod forms, mild hypochromia and some target cells. The plasma bilirubin level was normal The blood groups excluded the paternity of I,1 in his case: he was of the B group, whereas the tw parents and the other children had the O group It was concluded from the data that he had inherited elliptocytosis from his mother but it remains uncertain if this trait contributed



Fig. 2. Aseptic necrosis of the ribs in the propositus (II 8). Radiography taken 1 month after onset of the symptoms. Periosteal reaction, thickening of the bone and oval lacunae in the 5th and 6th ribs on the right.

Family Study

This family originates from the village of Ngungu, in the region of Gombe-Matadi of the Low Zaire Province. It belongs to the Besi Ngombe tribe, which is a part of the great Kongo ethnic group.

The parents and 6 male children are still alive (fig. 3, table I). 3 other female children died, one a few days after birth during convulsions of unknown origin, another in consequence of the sequelae of a *H. influenzae* meningitis and the third during an attack of acute malaria.

The *father* (I 1) is a β-thalassaemia trait carrier as indicated by hypochromic anaemia with high Hb A and normal serum iron (table I), by increased osmotic resistance (50% haemolysis at 0.35 g NaCl/dl and 100% at 0.10) and by numerous target cells and conspicuous poikilocytosis in the blood smears. He had also a hepatomegaly (1 cm).

The *mother* (I 2) is a slightly anaemic sickle cell trait carrier; besides, her blood smears showed about 70% of distinctly elliptic-shaped erythrocytes: she had consequently a hereditary elliptocytosis in association with the sickle cell trait. Neither any clinical abnormality nor signs of haemolysis were found. Hb F and Hb A were normal. Hb S was low (24.7%).

to his anaemia, which was normocytic but hypochromic and therefore possibly only due to ankylostomiasis.

The *junior brother* (II, 9) 4 years old, was in poor condition owing to chronic malaria, but had not inherited any of the traits carried by his parents.

Discussion

According to the extensive data collected in most of the ethnic groups of Zaïre and reviewed by LIVINGSTONE [16] and by HIERNAUX [11] the incidence of the sickle cell trait is high in this country: this is confirmed by the rates of prevalence found in the five main cities of Zaïre, which range from 23.7 to 29.4 [10, 29]. On the other hand, β-thalassaemia is much less frequent [8, 26] and its prevalence has been estimated at about 0.3% [29]. Consequently homozygosity for Hb S is very frequent in newborns (1.5–2% of all births) and sickle cell β-thalassaemia is necessarily rare (about 0.2‰). Assuming for Zaïre a population of 23,560,000 inhabitants with a natality rate of 44.4‰ [27], 15 000 births a year of SS homozygotes and 200 births a year of sickle cell β-thalassaemia (Hb S β-Th) carriers are acceptable estimations for this country, i.e. a proportion of one S-Th heterozygote for 75 SS homozygotes [31].

Two main haemoglobin phenotypes are found in case of Hb S β-Th. In the first type, termed 'sickle cell β^+ thalassaemia', Hb A is present in detectable amount because the β^A-chain synthesis is reduced but not suppressed by the thalassaemic mutation; the more often Hb A amounts to 20–30% of total haemoglobin, but much lower proportions were found in some cases [9, 20, 32, 35]. In the second type, the β^A-chain synthesis seems completely suppressed and no Hb A is found at electrophoresis or chromatography: this form, called sickle cell β^0-thalassaemia, simulates homozygous sickle cell anaemia; even the level of Hb F and of Hb A_2 of these patients overlap with those of the SS homozygotes in spite of their heterozygosity for β-thalassaemia [33, 39]. Both types coexist in many populations, their relative frequencies remain unknown, but the β^0-type was found more frequently than the β^+-type in Italians [24], in Jamaicans [37] and in Columbians [9]; in American Negroes the β^0-type was reported in 15 subjects and the β^+-type in 25 [19, 20, 35]. The racial distributions of each of both types probably differ, as it is the case in homozygous β-thalassaemia, of whom the β^+ type was only found in Northern Italy [6, 7], in Ghana [22] and in Thailand [34].

Table I Some haematologic data of the family members[1]

Case No[2]	Sex	Age years	Hb g/dl	PCV /l	MCHC g/dl	Serum iron µg/dl	Hb type	Hb A %	Hb F %	Hb A_2 %	Diagnosis
I 1	M	47	12.6	0.405	31.1	76	A	93.1	0.6	6.3	β-thalassaemia minor
I 2	F	42	11.7	0.37	31.7	–	A+S	71.9	0.5	2.9	sickle cell trait and hereditary elliptocytosis
II 1	M	25	16.4	0.49	33.4	116	A	96.7	0.6	2.7	hereditary elliptocytosis
II 3	M	22	14.0	0.45	31.1	91	A	97.0	0.5	2.5	malaria
II 4	M	20	12.0	0.42	28.5	65	A	94.7	0.4	4.9	β-thalassaemia minor and hereditary elliptocytosis
II 5	M	17	13.0	0.43	30.2	88	A	96.5	0.6	2.9	hereditary elliptocytosis, ankylostomiasis
II 8	M	7	9.8	0.33	29.6	67	S+A	33.6	4.2	5.8	sickle cell β-thalassaemia
II 9	M	4	10.4	0.35	29.7	–	A	96.5	0.5	2.7	malaria

[1] II 2, II 6, and II 7 = Deceased female children

[2] See figure 3.

severe pain which is not always ascribed to his true cause consequently their frequency might be underestimated [21]

On the occasion of the propositus family study examination of the mother showed she had not only the sickle cell trait but also hereditary elliptocytosis: she transmitted elliptocytosis to three of her sons, one of them (II 4) combining it with β-thalassaemia inherited from the father Elliptocytosis is not rare in Zafrian Bantus its incidence was estimated at 0.4 / [28] that is ten times the estimation proposed for the general population of the USA [40] therefore, cases of association of this trait with Hb S occur and some were described [14 28] In our case heterozygosity for the Hb S gene did not seem to interact with the expression of the elliptocytosis, this woman indeed had no sign of haemolysis and her slight anaemia was hypochromic: she did not differ in these respects from her two sons (II 1 and II, 5) who had elliptocytosis without Hb S. Also, no interaction was found in some other cases of this association documented by a family study [28] as well as in cases of association between elliptocytosis and Hb C [2]

One of the propositus brothers (II 4) combined elliptocytosis with β-thalassaemia he presented indeed typical thalassaemic abnormalities, including a high Hb A_2 level, whereas about 50% of his red cells were definitely elliptic, some of them being even rod-shaped ('bacteriocytes'). While the finding of a moderate number of elliptic erythrocytes is not uncommon in thalassaemia, these are rarely enough to cause difficulty in distinguishing this condition from hereditary elliptocytosis. In the son II 4 the proportion of the elliptic cells and the presence of strongly coloured rod-shaped forms make the double heterozygosity certain yet the data give no indication of enhancement of the effects of both traits: the abnormalities do not differ significantly from those of the father who had only β-thalassaemia This kind of association has been sporadically reported out of 6 reports mentioned by Aksoy and Erdem [1] mutual enhancement of the effects of the involved genes was described in 3, affecting on the whole five subjects. None of the characteristics resulting from the enhancement, i.e., an uncompensated haemolytic anaemia and in some cases an increase of Hb F were found in our patient, who therefore belongs to the group in which no interaction occurs. The possibility of the existence of two groups of cases in this kind of association, one with either gene interaction or enhancement of the effects of both traits and the other without interaction or enhancement, is easily conceivable: in elliptocytosis as in thalassaemia the phenotypic expression of the same gene in

In Zaïre many cases of Hb S β Th of both types were found, but in very unequal frequencies since 1956 indeed 47 cases of Hb S β^0 Th have been reported [15 26] whereas only two cases of Hb S β^+ Th were described [8 32] both were adults when diagnosed The first one born in Kasaï had complained since 7 years of age from painful osteoarticular crises the blood abnormalities involved a normosideremic microcytic anaemia, which needed only once a blood transfusion and a strongly diminished osmotic fragility besides Hb S he had about 3% Hb A the radiographies of the spine showed flattening of some dorsal vertebral bodies with central cupping and biconcavity ('fish vertebrae) The second patient [8] born in the Equator Province and aged 24 was hospitalized for a multilocular femoral osteomyelitis he had only a slight microcytic anaemia with normal serum iron and was never transfused the Hb A fraction amounted to 32%.

Since 47 cases of Hb S β^0 Th have been reported up to now and, including the present one only three of Hb S β^+ Th, the β^0-thalassaemia gene seems more frequent in Zaïrians than the β^+-gene especially when taking into account that the diagnosis of Hb S β^+ Th is generally easy when no blood transfusion was recently given it can indeed be easily suspected at the sole examination of the patient's electrophoretic pattern which shows Hb A in lesser amount than Hb S

It is generally admitted that the Hb S β-Th patients with Hb A have a milder clinical course than those without it [36] or at least present higher haematocrit values [20] many patients live to an old age and many others probably remain undiagnosed especially if they present 20–30% Hb A [15] This relative benignity results, at least partly from the low concentration of Hb S in their red cells, which in turn is due to the presence of Hb A and to the hypochromia associated with β-thalassaemia. Nevertheless the higher haematocrit values increase the blood viscosity so that the patients are more liable to ischemic tissue damage than to symptoms resulting from erythropoietic hyperactivity and chronic anaemia. Actually this was the case for the two Zaïrian adult patients previously described and also appears from the clinical course of our propositus, who had only one painful epidosde due to bone necrosis. The necrotic foci were exclusively localized at the ribs of both sides and were complicated by a pleuritis Such vaso-occlusive necroses of the ribs were very rarely reported in the sickle cell syndromes they were mentioned once in Zaïrians, in a review of the surgical pathology of sickle cell anaemia according to which they occur in infance and induce a moderate swelling and a more or less

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different members of the same family varies often largely and moreover different kinds of genes for elliptocytosis [4] and for β-thalassaemia [3] do exist so that interaction might arise in some cases of this association and not in others.

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G-6-PD Deficiency in the Newborn

Remarks on the articl of T. Meloni, S. Costa and S. Cutillo, Acta haemat 54 284–288 (1975): In the discussion part of the article, the authors very logically summarized their results as follows. 'This fact together with the irregular serum levels should lead to the conclusion that Hp determinations are not useful to evaluate the occurrence of hemolysis i newborn infants. This was reported previously by Rausen *et al.* [1]. Correlating the plasma hemoglobin levels with the presence or absence of haptoglobin in cord blood and in the newborn period, w reached the same conclusion more directly [2]. I spite of the bove statement, the following sentence was in the abstract. 'The findings confirm the authors' assumption expressed elsewhere, that this form of hyperbilirubinemia is not hemolytic in nature.' Since there is such discrepancy between these tw statements, I would like to question how much one could be certain about the presence or absence of hemolysis in the newborn period by the haptoglobin studies?

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40 WYANDT H BANCROFT P M and WINSHIP T O. Elliptic erythrocytes in man. Archs intern. Med 68 1043–1045 (1941)

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sicherten Tatsachen» Platz machen werden. Für jemanden, der eine einfache Einführung in die klinische Immunologie möchte, geht das Buch in zu viele Einzelheiten. Für jemanden, der die Vorgänge selbst begreifen möchte, machen die obengenannten Mängel das Buch wenig geeignet. Die Autoren besitzen eine weit gehende Übersicht über das Gebiet der klinischen Immunologie. Es ist schade, dass dieses Wissen nicht didaktischer genutzt wurde.

T. L. Vischer, *Genf*

Stacher, A., **Leukämien und maligne Lymphome**, Urban & Schwarzenberg, München 1973. XIX + 726 pp., 243 fig., 194 tab.

Im März 1972 wurde in Wien die 2. Internationale Arbeitstagung über Chemo- und Immunotherapie der Leukosen und malignen Lymphome abgehalten. Während bei der ersten Tagung 1969 die akuten Leukämien im Zentrum standen, waren nun die malignen Lymphome das Hauptthema. Die renommierten und auf dem Gebiet der malignen Lymphome gut ausgewiesenen Referenten vermochten eine umfassende *Inventar unseres Wissens vorzustellen. Schwerpunkt der Referate* waren pharmakologische und biochemische Aspekte der Wirkung verschiedener Zytostatika, Pathologie, Pathophysiologie und Zytologie maligner Lymphome, immunologische Veränderungen sowie Fragen der klinischen Diagnostik, Stadieneinteilung und Differentialdiagnose sowie der Therapie, insbesondere der Behandlung mit Zytostatikakombinationen. In Forumsdiskussionen wurden die angeschnittenen Probleme vertieft versucht, einheitliche Richtlinien für die Diagnostik und Therapie zu erarbeiten. Die vorliegende Monographie gibt eine hervorragende aktuell Standortbestimmung. Sie dokumentiert eindrucklich, dass beharrliche und systematische Anstrengungen besonders dann von Erfolg gekrönt sind, wenn sie interdisziplinär und auf dem Gebiet der Therapie kooperativ durchgeführt werden. Das Buch ist besonders Hämatologen und Onkologen zu empfehlen sowie allen onkologisch *interessierten Ärzten.*

J. P. Obrecht, *Basel*

G. Mathé and R. K. Oldham, ***Complications of Cancer Chemotherapy*** Recent Results in Cancer Research, vol. 49. Series Editor P. Rentchnick. Springer Berlin 1974. VI + 139 pp., 34 fig., DM 58, US $ 23.70

Die auf der Tagung der «European Organization for Research on Treatment of Cancer» (EORTC) im *Juni 1973 in Paris gehaltenen Vorträge zum Thema* «Komplikationen der Krebstherapie» werden nunmehr in Buchform vorgelegt. Die Toxizität von Zytostatika erstreckt sich über einen weiten Bereich, von der häufigen hämatopoetischen Depression und den Organstörungen bis zur noch nicht ganz geklärten immunologischen Toxizität. Als Ausdruck einer kombinierten hämatopoetischen und immunologischen Schädigung treten häufig infektiöse Komplikationen auf. Sie stellen den Kliniker immer wieder vor schwierige Probleme, denen er durch intermittierende Gaben von Zytostatika (die eine hämatopoetische und immunologische Erholung während behandlungsfreier Intervalle ermöglichen sollen), aseptische Umgebung (iff island) und Granulozytentransfusionen zu be

Book Reviews Buchbesprechungen Livres nouveaux

E. Grundmann (ed.) Special Topics in Carcinogenesis. Recent Results in Cancer Research vol. 44 Series Editor P Rentchnick. Springer Berlin 1974. VIII + 188 pp 54 fig. DM 58.–/US $ 22.40 ISBN 3-540-06460-5

Vom 24 bis 25 März 1972 veranstaltete die Gesellschaft zur Bekämpfung der Krebskrankheiten in Westfalen e. V ein Symposium über aktuelle Aspekte der Karzinogenese, an dem führende internationale Wissenschaftler teilnahmen. Die dort gehaltenen Referate erscheinen nun in Buchform. Sie behandeln zunächst allgemeine Probleme der Karzinogenese. Dabei werden molekularen Mechanismen der chemischen Karzinogenese und der Entstehung von Karzinogenen aus Vorläufern besondere Beachtung geschenkt. In einem weiteren Abschnitt kommen zytologische und histologische Veränderungen präkanzeröser und invasiver Läsionen sowie histochemische und elektronenoptische Probleme zur Erörterung. Zytogenetischen Schäden in der Karzinogenese im allgemeinen und Chromosomen-alterationen nach chemischen Karzinogenen, namentlich nach Diäthylnitrosamin, im besonderen ist ein weiteres Kapitel gewidmet. Schliesslich werden verschiedene Modelle der experimentellen Karzinogenese vorgestellt, z.B. sogenannte »minimal deviation tumors« unter Einfluss von Infektionen und Hormonen. Weiter wird die Rolle regionaler Lymphknoten in Entstehung und Wachstum von Tumoren oder Metastasen diskutiert.

Die Monographie gibt eine gute Übersicht über Teilaspekte der Tumorentstehung und ist experimentellen und klinischen Onkologen sowie allen an onkologischen Problemen interessierten Ärzten wärmstens zu empfehlen.

J P Obrecht Basel

Friedrich Scheiffarth und Hans-Wolf Baenkler Klinische Immunologie. 1975 XX + 330 pp 69 fig., 31 tab DM 46. ISBN 3-437 10394-6

Wie die Autoren im Vorwort schreiben, hat kaum ein Bereich im Grenzgebiet von Naturwissenschaften und Medizin einen so intensiven Entwicklungsprozess durchlaufen wie die Immunologie. Die kl nische Immunologie nimmt einen zunehmend breiten Raum in der Medizin ein. Das vorliegende Buch soll nun eine Lücke ausfüllen und dem Kliniker und dem praktisch tätigen Arzt die heute gesicherten Kenntnisse darstellen. Das Werk ist klar aufgebaut, fliessend geschrieben und deckt alle wichtigen Gebiete und Befunde der klinischen Immunologie. Zwei wichtige Mängel werden jedoch sichtbar. Der ganze St ff wird *ex cathedra* gegeben, wobei Argumente, Gründe und Beweise fast vollständig fehlen. Gleichzeitig wurde auf jegliche Literaturhinweise verzichtet. Die sogenannten gesicherten Tatsachen werden so jeder Nachprüfung entzogen. Und eine ganze Anzahl der gesicherten Tatsachen sind Arbeitshypothesen, die vermutlich in einigen Jahren anderen ge-

Acta haemat. 56 257–275 (1976)

Hb Helsinki a Variant with a High Oxygen Affinity and a Substitution at a 2,3-DPG Binding Site (β82 [EF6] Lys→Met)

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Key Words. 2,3-Diphosphoglycerate · Erythrocytosis · Hb Helsinki · Haemoglobinopathies · Haemoglobins with high oxygen affinity · Inositol hexaphosphate

Abstract. A new haemoglobin, Hb Helsinki, in which β82-Lys (EF6) is replaced by Met, was found in a Finnish family. It was associated with familial erythrocytosis, and the oxygen affinity of the blood was higher than normal. The oxygen equilibrium curves of purified Hb Helsinki and HbA from the same haemolysate have been determined under various conditions. 'Stripped' Hb Helsinki was found to show normal cooperativity, slightly low oxygen affinity and a reduced Bohr effect at physiological pH. However, the organic phosphates, 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (IHP) had a very small effect on Hb Helsinki, and the 2,3-DPG binding constant of deoxygenated Hb Helsinki is close to that of oxyhaemoglobin A. Thus, the replacement of Lys by Met at position 82 dramatically changes the nature of the central cavity of the tetramer and the effect of 2,3-DPG on the respiratory function of the molecule.

Familial erythrocytosis was first reported in 1908 by Nichamin [1]. It took 58 years to relate this phenomenon to a haemoglobin variant with a raised oxygen affinity, the first example of which was Hb Chesapeake described by Charache *et al.* [2]. Numerous examples are now known and it is usually possible to relate the change in function to a change in structure as summarised by Morimoto *et al.* [3]. Most of the early considerations of differences in structure related to the tetrameric $\alpha_1\beta_2$-contacts between two $\alpha\beta$-dimers which move during the reversible transition from oxy- to deoxy-haemoglobin [4]. The discovery of the role of organic

gegnen versucht Gegenüber diesen Frühnebenwirkungen gewinnen Spätnebenwirkungen der Chemotherapie immer mehr an Bedeutung Bekannt sind die Langzeitfolgen und namentlich die leukämogene Wirkung von Zytostatika (M. Hodgkin), vor allem wenn sie mit der Strahlentherapie kombiniert werden, ferner die Induktion von Sterilität bei jungen Patienten, während über genetische Schäden bei deren Nachkommen heute wenn überhaupt, kaum Material vorhanden ist. Grosse Anstrengungen werden experimentell und klinisch unternommen, um das Auftreten von Toxizitäten vorhersagen und rechtzeitig abschwächen zu können. Vor allem bei Langzeitbehandlungen, wie sie heute beim M Hodgkin und bei Non-Hodgkin-Lymphomen sowie beim Mammakarzinom durchgeführt werden, müssen diese Nebenwirkungen berücksichtigt und gegenüber dem therapeutischen Gewinn abgewogen werden. Zu all den angeschnittenen Problemen wurden auf dem Symposium beachtliche Referate gehalten. Sie geben einen ausgezeichneten Überblick über das weite Spektrum bekannter und noch wenig verstandener Nebenwirkungen der Krebschemotherapie Jeder onkologisch tätige Arzt sollte sie kennen. Er wird in der gut ausgestatteten Monographie über die Grundlagen wie über einzelne Erscheinungsformen der Nebenwirkungen bestens informiert.

J P Obrecht *Basel*

J H. Jepson and W S. Frankl, Hematological Complications in Cardiac Practice Saunders, London 1975 293 pp US $ –

This is an excellent interdisciplinary book dealing with cardiological implications of blood diseases and secondary blood disorders in cardiology The 17 authors are hematologists and cardiologists of worldwide reputation from USA and Canada The main contributions review cardiovascular manifestations of anemia hemodynamics in anemia polycythemia thalassemias, sickle cell disorders and abnormal hemoglobins affecting oxygen delivery 2,3-DPG blood disorders due to aortic valve prosthesis, infective endocarditis and cardiac drugs thrombogenesis and coagulolysis anticoagulation and its laboratory control unusual cardiac manifestations of hematological disease blood banking problems in cardiology cardiovascular dynamics and oxygen delivery in pregnancy The book can be recommended to every hematologist and cardiologist in clinical practice.

H R. Marti, *Aarau*

Table I Laboratory results obtained in 2 members of the propositus' family

Case	Age	Sex	Haemoglobin g/dl	RBC 10 /l	PCV l/l	WBC 10^9/l	Reticulocytes %	Platelets 10 /l	Red cell volume ml/kg	Serum iron µmol/l	TIBC µmol/l	Bone marrow iron	2,3-DPG mmol/l 25°C	P50, mm Hg* arterial	P50, mm Hg* venous
II	65	F	16.0		0.48										
II	53	F	14.0	5.08	0.41	6.3	2.6	19.8	26.2	15.0	69.8	depleted	3.4	93	23
III	29	F	16.4		0.51										
III	33	F	16.8		0.49										
III	26	M											3.0		
III	5	M	16.6	6.05	0.49	9.6	2.0	17.2	26.9	15.7	90.3	depleted	3.6	92	
Normal range		F	14.0±2.5	4.8 ±1.0	0.42±0.05	7.5±3.5		150–400	25±5	13–32	45–70				
		M	15.5±2.5	5.5 ±2.5	0.47±0.07	7.5±3.5		150–400	30±5	13–32	45–70				

1 KP = 7.5 mm Hg.

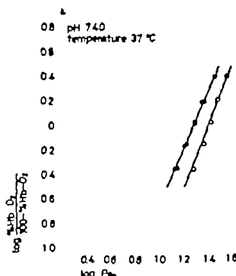


Fig. 2. Oxygen dissociation curve of whole blood containing Hb Helsinki (●) in the logarithmic form of Hill's equation compared to that of normal adult blood (O). Hill's coefficients: whole blood containing Hb Helsinki 2.30, normal adult blood 2.53, Severinghaus standard curve 2.61.

Case Report

The propositus, aged 36, was referred to the University Central Hospital of Helsinki because of a high haemoglobin level in his blood which was noted when he consulted his physician because of acute chest pain. No signs of an organic heart disease were found. On examination, he showed no plethora on his face and no cyanosis. His spleen was not palpable. No evidence of pulmonary disease was found. Laboratory results: haemoglobin 19.1 g/dl, packed cell volume (PCV) 0.541 l/l, reticulocyte count 3.9‰, white blood cell (WBC) count 6.1×10^9/l, platelet count 140×10^9/l, red cell volume 39.3 ml/kg. No free tissue iron was found in the bone marrow. Intravenous pyelography was normal. Arterial PO_{50} 72 mm Hg, venous PO_2 25 mm Hg (fig. 1, 2). The results obtained in other members of the family who were affected are shown in table I and the family pedigree is shown in figure 3.

In addition, 2 other men were found to have erythrocytosis due to Hb Helsinki. One of them, aged 67 years, had coronary heart disease and atrial fibrillation. Haemoglobin 19.6 g/dl, red cell volume 50.8 ml/kg, PO_{50} 19 mm Hg. The other carrier of Hb Helsinki was a 33-year-old blood donor with a haemoglobin level of 17.6 g/dl, red cell volume 32.9 ml/kg and PO_{50} 21 mm Hg. Thin-layer isoelectric focusing disclosed the same abnormal haemoglobin in 2 brothers of the latter but in none of the 3 children of the former.

No definite connections were found in 5 generations between these 3 families but some of the ancestors of the 1st and 3rd family have lived in the same small parish in South Western Finland at the beginning of the 18th century.

Table II Amino acid composition of $\beta^{Helsinki}$TpIX Xa

Amino acid	βTpIX Xa yield			β TpIX-Xa, number of residues
	quantity nmol	molar ratio	number of residues	
Asp	20.9	3.2	3	3
Thr	10.9	1.6	2	2
Ser	10.7	1.6	2	2
Glu[1]	8.6	1.3	1	1
Gly	21.0	3.2	3	3
Ala	18.3	2.8	3	3
Val	5.4	0.8	1	1
Met	5.4	0.8	1	
Leu	42.5	6.4	6	6
Phe	14.5	2.2	2	2
Lys				1

Contaminated with homoserine.

Table III Amino acid composition of CB fragment $\beta^{Helsinki}$CB56–82

Amino acid	$\beta^{Helsinki}$CB56–82 yield			β^{A}56–82, number of residues
	quantity nmol	molar ratio	number of residues	
Asp	25.7	4.2	4	4
Ser	5.4	0.9	1	1
Pro	5.1	0.8	1	1
Gly	26.0	4.3	4	4
Ala	17.0	2.8	3	3
Val	10.0	1.7	2	2
Leu	24.3	4.0	4	4
Phe	5.7	0.9	1	1
His	11.2	1.9	2	2
Lys	25.6	4.3	4	5
Homoserine and homoserine lactone[1]		detected		

Contaminated with homoserine lactone.
Interconversion takes place on the analyser column [32].

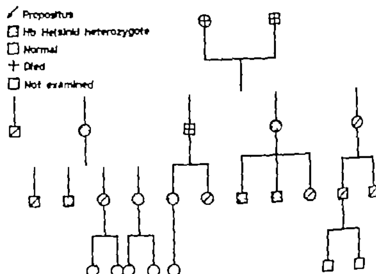


Fig 1 Pedigree of the propositus.

Results

Identification of the abnormal haemoglobin. Paper and starch-gel electrophoresis at pH 8 9 and 8.2, respectively showed a fast-moving haemoglobin Diagnostic chain separation of globin on cellogel revealed a fast moving β-chain Thin layer isoelectric focusing disclosed the same abnormal haemoglobin in 6 other members of the family Comparison of the finger prints of normal and abnormal AE β-chains showed that, in the latter the peptides βTpVIII IX (β66-82), βTpXa (β83-93) and βTpX (β83-95) were missing (fig. 4) A peptide migrating in the position of βTpIX reacted with reagents specific for both His and Met whilst $β^A$TpIX contains His only and no Met Furthermore, material which had remained at the origin during electrophoresis and had streaked on chromatography also reacted with both the His and the Met reagents. On electrophoresis of the streak at pH 3.5 the major peptide recovered on analysis had a similar composition to $β^A$TpIX + $β^A$TpXa (table II) except that the residue of lysine was missing and that there was an additional residue of methionine. The positive staining reaction with the reagent for divalent sulphur and the presence of the methionine residue in βTpIX X suggested a replacement of a Lys by a Met. Chemical cleavage of the normal and abnormal AE β-chains using CB confirmed this assumption. A CB

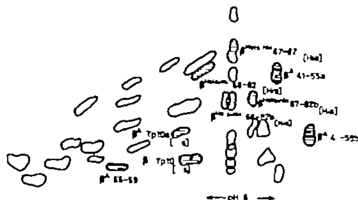


Fig. 6. Line diagram of the 'finger-print' of tryptic peptides derived from the CB fragments (β1 55, 56-82 and 83-146 of AE βHelsinki chain. For experimental conditions, see legend to figure 4.

with carboxy-terminal methionine residues which had during CNBr cleavage been converted to homoserine and homoserine lactone (table IV). Thus, these results confirm that the mutation is β82Lys-Met.

Functional properties of Hb Helsinki. The methaemoglobin concentration was less than 3% in all cases. The oxygen equilibrium curves of Hb Helsinki and HbA were determined at 4 different pH values. Hill plots of HbA and Hb Helsinki are shown in figures 7a, b. In both cases, the 4 curves determined at different pH values converge at the upper extremities implying that the 4th Adair constant (K_4) is independent of pH [33]. As shown in figures 8a, b the oxygen-binding properties of stripped Hb Helsinki are very similar to those of HbA. Hb Helsinki has normal cooperativity throughout the pH range examined and has a normal oxygen affinity at pH 6.8, but the oxygen affinity is lower than for HbA at higher pH and Hb Helsinki consequently has a slightly reduced Bohr effect. When the cooperativity is very high, K_1 and K_4 are very close to $1/K_T$ and $1/K_R$ [34] respectively i.e. the oxygen-binding constants for T and R structures [35]. In the case of Hb Helsinki, the oxygen affinity of the T structure (K_T) is close to normal, but the oxygen affinity of the R structure (K_R) is significantly lower than normal by 0.2 log unit. Table V summarises the oxygen-binding parameters of Hb Helsinki and HbA in the absence and in the presence of organic phosphates and also includes data on Hb Rahere in which β82Lys is replaced by Thr [36]. DPG strikingly

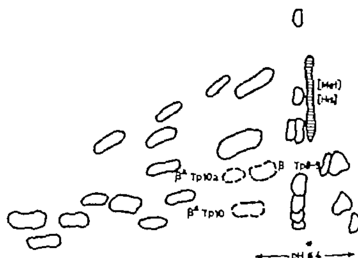


Fig 4 Line diagram of the 'finger-print' of the tryptic peptides of the AE β-chain from Hb Helsinki. Electrophoresis at pH 6.4 (60 V/cm, 1 h) ascending chromatography in the upper phase of pyridine-isoamyl alcohol-water (6:6.7 by vol), for 20 h. Peptides were located using 0.2% (w/v) ninhydrin in acetone containing 1 (v/v) pyridine. Broken lines indicate missing peptides. The shaded spot indicates the new peptide. ▬ Origin.

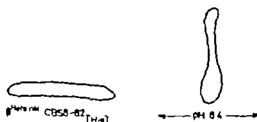


Fig 5 Line diagram of the 'finger print' of the CB peptide (β56–82) of Hb Helsinki. For experimental conditions, see legend to figure 4

fragment, which gave a specific staining reaction for His, was detected in a finger print of the abnormal chain only (fig. 5) Analysis showed this CB peptide to be identical with β 56–81 (table III) but also to contain homoserine and homoserine lactone. Tryptic digestion of the CNBr treated AE β-chains followed by finger printing, revealed a number of abnormal peptides (fig 6) The amino acid analyses obtained for these peptides were consistent with those expected for βTpVIII IX and βTpIX

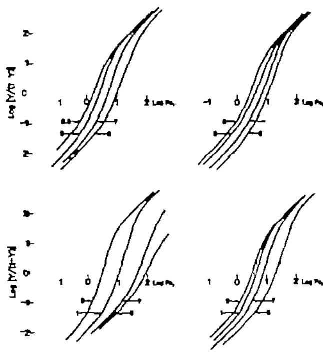


Fig 7 Oxygen-binding curves (Hill plots) for Hb Helsinki and HbA. Haemoglobin concentration, 60 µM, 25 °C in 0.05 M bis-Tris 0.1 M Cl (below pH 7.4) or in 0.05 M Tris 0.1 M Cl^- (above pH 7.9). HbA (stripped). *b* Hb Helsinki (stripped). HbA with 2 mM IHP *d* Hb Helsinki with 2 mM IHP

lowers the oxygen affinity of HbA, but this has practically no effect on Hb Helsinki.

The effect of inositol hexaphosphate (IHP) has been investigated in more detail. Figures 7c, d show Hill plots for HbA and Hb Helsinki in the presence of 2 mM IHP and the oxygen-binding parameters for the same data are shown in figures 8c, d. IHP very strongly binds to HbA and pulls the allosteric equilibrium towards the T-deoxy structure and reduces cooperativity down to about n = 2 at pH 6.8. Under these conditions, even K_t becomes strongly dependent upon pH. On the other hand, in the case of Hb Helsinki, neither PO_{50} nor K is greatly affected by addition of IHP above pH 7.4. However at pH 6.8 IHP lowers log PO_{50} by 0.25 and even K_t is slightly affected by IHP

Table IV Amino acid composition of $\beta^{Helsinki}$66–82 abnormal tryptic peptide formed from CB fragments of the AB β-chain

Amino acid	Tp fragment composition, molar ratio				Expected composition, number of residues	
	66–82a[1]	66–82b[2]	67–82a[3]	67–82b[4]	β^A66–82	β^A67–82
Asp	2.9	2.9	2.9	2.9	3	3
Ser	1.0	1.1	0.9	1.0	1	1
Gly	2.3	2.2	2.1	2.0	2	2
Ala	2.2	2.3	1.8	2.1	2	2
Val	1.1	1.3	1.0	0.9	1	1
Leu	3.6	4.3	4.1	3.8	4	4
Phe	1.0	1.3	0.8	0.9	1	1
His	1.0	1.1	1.2	1.1	1	1
Lys	0.8	0.8	–	–	2	1
Homoserine and homoserine lactone	detected		detected		–	

[1] Residue = 2.3 nmol.
[2] Residue = 1.0 nmol.
[3] Residue = 1.6 nmol.
[4] Residue = 2.4 nmol.

Table V The effect of amino acid substitutions at position β82 (EF6) on the PO_{50} and n values of haemoglobin

	Hb Helsinki β82Met		Hb Rahere β82Thr		HbA, β82Lys[1]	
	PO_{50}, mm Hg	n	PO_{50}, mm Hg	n	PO_{50}, mm Hg	n
Stripped	7.03	3.08	3.80	reduced (2.10)	5.01	3.07
2 mM DPG	7.94	3.09	5.67	normal	14.29	3.05
2 mM IHP	8.51	3.10	9.99	normal	51.2	2.59

Experimental conditions 25 °C in 0.05 M bis-Tris 0.1 M Cl^- pH 7.25–7.3.
[1] Taken from Lorkin *et al.* [36].

Table VI Effect of DPG on various haemoglobins

		Stripped		DPG		PO_{50} (DPG) / PO_{50} (str)	DPG	Cl^-	pH	log K_4	References
		PO_{50} mm Hg	n	PO_{50} mm Hg			mM	M		M^{-1}	
Hb Helsinki	β82Lys-Met	7.03	3.08	.94	3.09	1.13	2.0	0.1	7.3	2.53	present study
Hb Rahere	β82Lys-Thr	3.80	2.1	5.67	2.8	1.49	2.0	0.1	7.25	3.46	[36]
HbA		5.01	3.07	14.29	3.05	3.02	2.0	0.1	7.3	4.76	present study
Hb Little Rock	β143His-Gln	0.82		1.6		1.95	0.62	0.1	7.2	3.36	[42]
HbA		2.79		6.57		2.41	0.62	0.1	7.2	4.77	[42]
Hb Syracuse	β143His-Pro	0.33	1.1	0.51		1.35	1.0	0.1	7.2	3.77	[43]
HbA		2.78		7.51		2.7	1.0	0.1	7.2	4.79	[43]
Hb Abruzzo	β143His-Arg	0.80	0	8.79		11.0	5.0	none	7.0		[45]
HbA		1.80	2.4	12.60		7.0	5.0	none	7.0	–	[45]
HbF	β143His-Ser	1.88	2.65	2.25	2.85	1.20	0.2	0.1	7.4	3.76	[7]
HbF	β143His-Ser (N terminus acetylated)	2.33	2.45	2.38	2.35	1.02	0.2	0.1	7.4	2.79	[7]
HbA		1.29	2.4	2.72	2.45	2.10	0.2	0.1	7.4	4.95	[7]
HbA_1	hexose attached to N terminus	1.06	2.4	1.31	2.4	1.23	0.2	0.1	7.4	3.83	[7]
$\alpha_2\beta_2^c$	N terminus carbamylated	5.97	3.04	8.2	2.89	1.37	0.5	0.1	7.3	3.76	[44]
HbA		4.15	2.63	11.5	2.32	2.77	0.5	0.1	7.3	5.10	[44]
Hb Deer Lodge	β2His-Arg	0.62	2.0	4.90	2.7	7.90	1.0	none	7.4		[46]
HbA		1.00		8.53		8.53	1.0	none	7.4		[46]

All the measurements were carried out in 0.05 M bis-Tris buffer

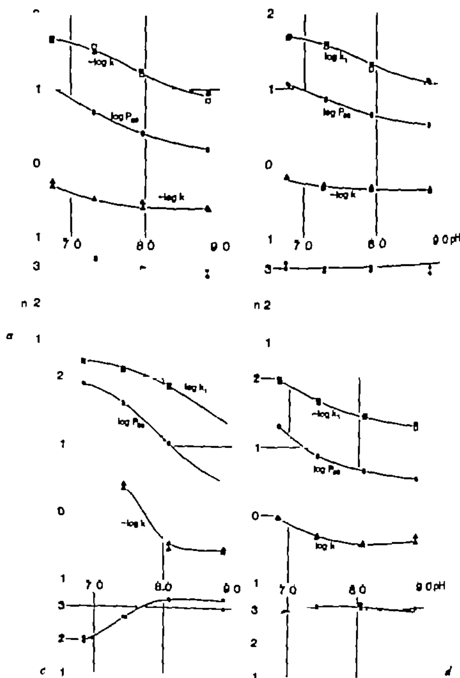


Fig. 8. Oxygen-binding parameters of stripped HbA (a) Hb Helsinki (b), HbA with 2 mM IHP (c) and Hb Helsinki with 2 mM IHP (d). The parameters were graphically determined from the Hill plots, and two sets of points were determined from both deoxygenation and reoxygenation curves. Experimental conditions as in figure 7a–d.

(HbF) has a smaller 2,3-DPG effect compared with HbA and this could be explained by the substitution of Ser for β143(H21)His. In HbF log B_o is decreased by 1.19 and further decreased by 0.97 in HbF_{1a} in which the α-amino group of the N terminus is acetylated. In the case of HbA_{1c}, the loss of a positive charge from β1Val through the attachment of hexose to the α-amino group results in a decrease of log B by 1.12 [4] If the same α-amino group is carbamylated ($\alpha_2\beta_2$) [44] the log B_o value is decreased by 1.4 The results on Hb Abruzzo β143His-Arg [45] and Hb Deer Lodge β2His-Arg [45] do not permit the estimation of log B values as the experiments were carried out in the absence of 0.1 M Cl However comparing their PO_{50} (DPG)/PO_{50} (str) values with that of HbA, it can be concluded that the replacement of His by the positively charged group did not alter the 2,3-DPG binding constants significantly Therefore, in all cases the loss of the positive charge of the basic groups of β1Val, β143His or β82Lys reduced log B by 1.97–1 4 In Hb Helsinki, the corresponding value is a decrease by 2.23 Furthermore, IHP changes log PO_{50} for Hb Helsinki by only 0 08 at pH 7.3 as shown in tables V and VI. Using the IHP binding constant obtained for oxyhaemoglobin ($\times 10^{5}$ M^{-1}) [47] the IHP binding constant for deoxyhaemoglobin Helsinki is calculated to be 2.2×10^{5} M^{-1} from equation (1), indicating that the IHP binding constant for Hb Helsinki remains almost unchanged on deoxygenation. In Hb Helsinki, the nature of the substitution rendered the central cavity of the molecule inaccessible for both 2,3-DPG and IHP This may affect the groups responsible for the alkaline Bohr effect and reduce the effect correspondingly

Although the experimental conditions are not quite the same as in the blood cells *in vivo*, it is possible to use the results to estimate how Hb Helsinki might contribute to the transport of oxygen to the tissues under physiological conditions. The percentage oxygenation of haemoglobin in normal red cells is estimated to be about 95% in the arterial and 73 / in the venous blood. The oxygen partial pressures at which HbA under experimental conditions in the presence of 2,3-DPG is 95 and 73 / oxygenated were calculated. Under the same conditions, the corresponding percentage oxygen saturation of Hb Helsinki, permits the release of 5 4 /o and compared with HbA would deliver a quarter only to the tissues. It is conceivable that this causes the compensating erythropoiesis.

The propositi of the 3 families with Hb Helsinki were found within 2 years among the patients with unexplained erythrocytosis at the University Central Hospital of Helsinki. During the preparation of the manuscript

Discussion

2,3-DPG exerts two regulatory mechanisms on oxygen binding to haemoglobin molecules It diminishes haemoglobin oxygen affinity through the Bohr effect by reducing intracellular pH relative to plasma pH and by preferential binding to the deoxy formation of haemoglobin [37] Alterations to the binding sites of this phosphate ester result in increase in haemoglobin affinity for oxygen secondary to the decrease in its affinity for 2,3 DPG

Table VI shows the oxygen-binding parameters of various haemoglobins in which particular residues involved in DPG binding have been altered either by mutations or by chemical modifications. All the PO_{50} (DPG)/PO_{50} (str) values cannot be compared directly as the experiments have been carried out at different DPG concentrations. However the values of B (DPG binding constant for fully deoxygenated haemoglobin) can be evaluated using BALDWIN's [34] equation (1). On addition of DPG, the change in P_m (medium ligand activity) [38] is related to the DPG binding constants for deoxyhaemoglobin (B_o) and oxyhaemoglobin (B_4) by the following equation

$$\log PO_{50\,(DPG)} - \log PO_{50\,(str)} = 1/4\ [\log(1 + B_o\,[DPG]) - \log(1 + B_4\,[DPG])] \quad (1)$$

P_m is very close to PO_{50} and can be replaced by PO_{50} in equation (1). BALDWIN [34] estimated B_4 to be $2.2\times10^{3}\ M^{-1}$ and this value is very close to the experimentally determined value of $2.5\times10^{3}\ M^{-1}$ [39] Although the 2,3-DPG and IHP binding sites on oxyhaemoglobin have not yet been identified, it is assumed that the central cavity of oxyhaemoglobin is too narrow to accommodate the 2,3-DPG or IHP molecule. They may bind to oxyhaemoglobin in a different manner because on addition of 2,3 DPG or IHP the number of protons released and pH dependence differ between deoxygenated and oxygenated structures [40 41]

It is expected therefore, that the binding constant of 2,3-DPG to oxyhaemoglobin is insensitive to structural alterations in the central cavity It is on this basis, that log B values have been calculated (table VI). B_4 values calculated for HbA agree with previous experimental results, implying that the B_4 value here is not greatly abnormal. It can be seen that log B for Hb Helsinki is decreased by 2.23 and is very close to log B_4 of 2.34 log B for Hb Rahere β82Lys-Thr is decreased only by 1.30 in Hb Little Rock β143His-Gln [42] by 1 41 in Hb Syracuse β143His-Pro by 1 02. BUNN and BRIEHL [4] showed that human fetal haemoglobin

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yet another family with erythrocytosis and a Hb Helsinki band on electrophoresis has been found in Western Finland It can be assumed that this abnormality which so far has not been found in any other population, might be relatively common amongst Finns. The gene is perhaps more frequent in the Western part of Finland where the early settlers lived as pointed out by NEVANLINNA [48] and the mutation has probably arisen earlier rather than more recently in the Finnish population.

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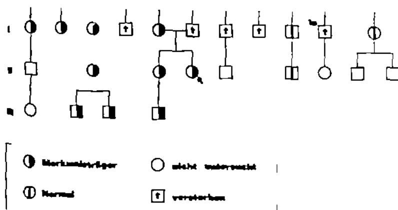


Abb 1 Stammbaum der Hämoglobin-E-Trägerin (Pfeil): Die Kreise bezeichnen die weiblichen, die Quadrate die männlichen Familienmitglieder

In der vorliegenden Arbeit berichten wir über eine in Süddeutschland ansässige Familie, in der bei 10 Mitgliedern ein heterozygotes HbE gefunden wurde. Anlass zur Durchführung der Hb-Analyse war eine deutliche Hypochromie der Eryhtrozyten bei normalem Serumeisen, so dass klinisch der Verdacht einer heterozygoten β-Thalassämie bestand.

Kasuistik

Im Juli 1973 wurde eine 37jährige deutschstämmige Patientin (Abb 1 II-4) wegen einer radiologisch objektivierbaren Wassermann-positiven Viruspneumonie aufgenommen. Sie gab Atemnot, Schmerzen beim Durchatmen und spärlich zähen Auswurf mit fadenförmigen Blutbeimengungen an. Bei der klinischen Untersuchung fielen lediglich vereinzelte kleine weiche mobile Lymphknoten beiderseits nuchal und eine mäßig ausgeprägte Struma diffusa auf.

Unter den Laborbefunden imponierte (bei Normalwerten für BKS, Leukozyten, Thrombozyten- und Retikulozytenzahl, Bilirubin, LDH sowie Haptoglobin) eine Hypochromie von 26 pg, die sich aus 12,2 g% Hb und 4,6 Mio. Erythrocyten errechnete, bei normalem, bei Kontrolle sogar erhöhtem Serumeisen (74 bzw. 191 und 204 µg%). Im Blutausstrich leichte Hypochromie und Anisozytose der Erythrozyten. Diese Befundkonstellation liess differentialdiagnostisch an eine Thalassämie denken.

Entsprechende Untersuchungen wurden auf die Familie ausgedehnt. Bekannt eren 18 lebende Blutsverwandte. 12 davon konnten auf das Vorliegen einer Hämoglobinanomalie untersucht werden (Abb. 1): bei 9 Angehörigen der mütter

Acta haemat. 56 276–284 (1976)

Hypochromie der Erythrozyten bei heterozygotem Hämoglobin E (β26 Glu→Lys)

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Key Words Erythrocyte hypochromia Haemoglobin E Haemoglobinopathies

Abstract A family from south-western Germany with 9 heterozygous carriers of haemoglobin E (β26 Glu Lys) is presented. The abnormal haemoglobin has been identified by different electrophoretic techniques and fingerprint analysis. In contrast to earlier observations, haemoglobin E is even in heterozygous carriers associated with erythrocytic hypochromia with MCH values from 23 6 to 6.2 pg As an explanation for the hypochromia a decreased haemoglobin synthesis is supposed.

Hämoglobin E wurde von Itano *et al* [16] und gleichzeitig von Chernoff und Minnich [9] bei der Abklärung einer hämolytischen Anämie entdeckt. Es gehört zu den häufigsten anomalen Hämoglobinen. Hauptverbreitungsgebiet ist Südostasien wo bis zu 35% der Bevölkerung Anomalieträger sind [8] Einzelfälle wurden in der Türkei, in Griechenland Dänemark [30] Italien [2, 25] Japan [28] im Kongo [14], in den USA [26] und in Kanada [30] beobachtet In Deutschland wurde eine Familie mit drei heterozygoten HbE Trägern von Betke und Kleihauer [6] beschrieben HbE besitzt nur im homozygoten Status oder als HbE/β-Thalassämie (doppelte Heterozygotie) eine pathogenetische Bedeutung, die sich als hämolytische Anämie mit verkürzter Erythrozytenlebenszeit Splenomegalie und Target Zellen im peripheren Blutbild manifestiert Bei heterozygoten HbE Trägern waren bisher keine hämatologischen Veränderungen bekannt [10 14 20] Aufgrund neuerer Untersuchungen von Paonier *et al* [23] ist die Hämoglobinsynthese jedoch vermindert.

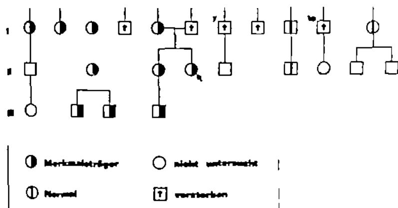


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Unter den Laborbefunden imponierte (bei Normalwerten für BKS, Leukozyten-, Thrombozyten- und Retikulozytenzahl, Bilirubin, LDH sowie Haptoglobin) eine Hypochromie von 26 pg, die sich aus 12,2 g% Hb und 4,6 Mio. Erythrocyten errechnete, bei normalem, bei Kontrolle sogar erhöhtem Serumeisen (74 bzw. 191 und 204 µg%). Im Blutausstrich leichte Hypochromie und Anisozytose der Erythrozyten. Diese Befundkonstellation ließ differentialdiagnostisch an eine Thalassämie denken.

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Tabelle I Hämatologische Daten von 9 Merkmalsträgern (II-4 = Proposita)

Familien mitglied	Geschlecht	Alter Jahre	Hb g%	Erythro-zyten Mio	MCH pg	Retikulo-zyten /oo	Serum-eisen µg %	Bilirubin mg%	LDH mU/ml
I 1	w	64	12,8	4 87	26,2		139	0,9	230
I 2	w	60	13 1	5 06	25 7		32	1 6	196
I 3	w	63	13 9	5,56	[illegible]4 8		170	1,2	146
II	w		13,2	5,26	[illegible]5 0		101	2,8	141
II 3	w	38	13 4	5,24	25,3			0,6	160
II-4	w	37	12,2	4 64	[illegible]6,0	1[illegible]	191	0,5	122
III 2	m		14 9	6,07	[illegible]4 4		40	1,2	200
III 3	m		14 0	5 73	24 2		101	0,6	12[illegible]
III-4	m	8	1[illegible],6	5,30	23 6		111	0,9	175

lichen Linie konnte eine Hämoglobinopathie nachgewiesen werden Die hämatologischen Parameter sind in Tabelle I zusammengestellt. Bei allen fallen die erniedrigten Hb_E Werte bei normalen Hämoglobinkonzentrationen auf. Die vereinzelt pathologisch veränderten Eisen- Bilirubin und LDH Werte wurden bei den weit über Süddeutschland verstreut wohnenden Probanden nicht weiter abgeklärt.

Methoden

Herstellung von Hämolysaten durch Wasserhämolyse gewaschener Erythrozyten und Extraktion in Tetrachlorkohlenstoff HbF Bestimmung zytologisch mit der Elutionsmethode [17] quantitativ mittels Alkalidenaturierung [3]. Hämoglobinelektrophorese zur quantitativen Bestimmung normaler und anormaler Hb-Fraktionen auf dem Stärkeblock bei pH 8 6 [4] auf der Mikrozonenelektrophorese im diskontinuierlichen TEB NaOH Puffersystem [18] ferner bei pH 6,2 auf Zellulose-Acetat Membranen, die mit Citratagar beschichtet waren [27] Hitzedenaturierung als Oxyhämoglobin bei 50 °C [12] sowie als Zyanmethämoglobin bei 65 °C [5]. Globinpräparation durch Hämabspaltung im eiskalten Aceton-Salzsäure-Gemisch [1]. Präparative Isolierung der Polypeptidketten durch Säulenchromatographie auf Karboxymethylzellulose CM , [11]. Fingerprintanalyse tryptisch gespaltener Polypeptidketten auf DC Fertigplatten-Kieselgel 60 (Merck AG Darmstadt) [19]

Ergebnisse

Hämoglobinanalyse Auf der Elektrophorese trennte sich aus dem Hämolysat der Proposita sowohl im Stärkeblock als auch auf Zellulose-

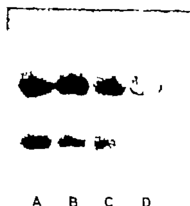


Abb 2. Elektrophoretische Trennung (Mikrozonenelektrophorese) von Hämolysat der Proposita (A), deren Mutter (B) und Schwester (C) daneben normal Kontrolle (D).

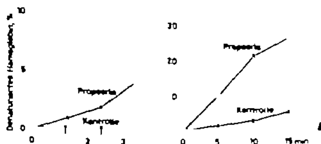


Abb 3 Vergleich der Hitzelabilität vom Gesamtblutfarbstoff der Proposita und von normalem HbA. Oxyhämoglobin bei 50 C. *b* Zyanmethämoglobin bei 65 C.

Acetat-Folien eine langsam wandernde anomale Hb-Fraktion ab, deren quantitative Bestimmung 45% vom Gesamtblutfarbstoff ergab (Abb 2). Aufgrund der Position im Elektrophoresediagramm handelte es sich um HbE oder HbC. Die Differenzierung auf mit Citratagar beschichteten Zellulose-Acetat-Folien ergab Identität mit HbE, welches mit dieser Methode wie HbA_2 wandert, während HbC anodenwärts von HbA zur Darstellung kommt [25]. Von den 12 untersuchten Familienmitgliedern wiesen 9 ebenfalls ein abnormes Hb in Mengen zwischen 38 und 45%

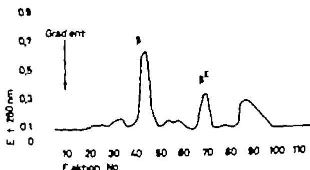


Abb 4 Säulenchromatographische Kettentrennung von Gesamtglobin der Proposita auf CM $_\gamma$. Die abnorm strukturierte Kette ist mit β^E gekennzeichnet.

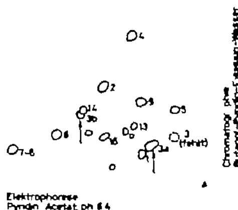


Abb 5 Fingerprint Anstelle des fehlenden Peptides βTp3 sind an anderen Stellen (▲) zwei neue Peptide βTp3a und βTp3b nachweisbar △ O = Auftragungsstellen.

auf (Abb 1) HbF war mit 0 7‰ nicht vermehrt in Ausstrichpräparaten waren keine HbF Zellen vorhanden

Stabilitätstests Das Gesamthämoglobin der Proposita zeigte als Oxyhämoglobin bei 50 C und als Zyanmethämoglobin bei 65 C eine deutlich höhere Hitzelabilität im Vergleich zum Hämoglobin einer normalen Kontrollperson (Abb. 3)

Strukturanalysen Nach Abspaltung des Hämanteils trennte sich bei der chromatographischen Kettentrennung aus dem Gesamtglobin der Proposita eine anomale β-Kette ab der Strukturdefekt betraf somit die β Kette (Abb 4) Das Protein der abnormen β Kette wurde tryptisch ge-

spalten und das Peptidgemisch zweidimensional mittels Elektrophorese und Chromatographie getrennt. Im Vergleich zum Fingerprint der normalen β-Kette zeigte die β^E-Kette ein abweichendes Muster (Abb. 5): Infolge des Austausches von Glutamin in Position 26 durch Lysin [15] entstanden aus dem Peptid βTp3 zwei Bruchstücke, die im Fingerprint als βTp3a bzw. 3b zur Darstellung kamen. Mit diesen Veränderungen im Fingerprintmuster wurde das anomale Hämoglobin der Proposita eindeutig als HbE identifiziert.

Diskussion

Hämoglobin E gehört zu den klinisch harmlosen Hb-Varianten. Es ruft erst bei homozygoter Anlage oder bei doppelter Heterozygotie mit der β-Thalassämie hämatologische Veränderungen hervor. Heterozygotie gilt als symptomlos, ohne Auswirkungen auf Blutbild und Erythrozytenmorphologie. Die Auffindung heterozygoter Individuen bleibt dementsprechend meist systematischen Studien oder dem Zufall überlassen, hat aber ihre Bedeutung für genetische Fragen und zur Vermeidung diagnostischer Irrwege. Der anomale Blutfarbstoff wird in Mengen zwischen 25 und 35% vom Gesamthämoglobin gebildet, bei Eisenmangel wird HbE in weit geringerem Masse synthetisiert [31]. Der Aminosäurenaustausch in Position 26 der β-Kette liegt in der B-Helix, wodurch die Stabilität des Kontaktes zwischen α1 und β1 Ketten gestört wird. Damit lässt sich möglicherweise die erhöhte Hitzelabilität erklären, die vermutlich auf einer verminderten Affinität von normalen α-Ketten zu β^E-Ketten mit folgender Dissoziation der $\alpha\beta$-Dimere in Monomere beruht [24].

Bei normalem oder erhöhtem Serumeisen wurde eine Hypochromie der Erythrozyten mit erniedrigtem MCH bisher nur bei Homozygotie für HbE beschrieben [10]. Dies gilt mit der Einschränkung, dass Lie Injo *et al.* [21] MCH-Werte von 25,3–26,5 pg bei Patienten mit HbE kombiniert mit hereditärer Elliptozytose feststellten. Demgegenüber hatten alle Hämoglobin-E-Träger der hier beschriebenen Familie bei heterozygoter Anlage eine Hypochromie mit MCH-Werten zwischen 23,6 und 26,2 pg. Identische Befunde wurden neuerdings bei drei Mitgliedern einer HbE-Familie aus Aachen erhoben, die MCH-Werte zwischen 22,9 und 25,6 pg aufwiesen [unveröffentlichte Befunde].

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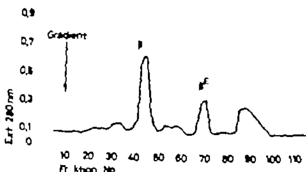


Abb 4 Säulenchromatographische Kettentrennung von Gesamtglobin der Proposita auf CM $_F$. Die abnorm strukturierte Kette ist mit βE gekennzeichnet.

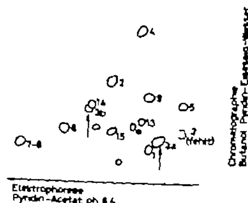


Abb 5 Fingerprint Anstelle des fehlenden Peptides βTp3 sind an anderen Stellen (↑) zwei neue Peptide βTp3a und βTp3b nachweisbar △ O = Auftragungsstellen.

auf (Abb. 1) HbF war mit 0 7 % nicht vermehrt, in Ausstrichpräparaten waren keine HbF Zellen vorhanden

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Trägern eine erniedrigte Synthese von HbE mit einem Überschuss an freien α-Ketten und vermuteten eine verminderte oder verzögerte Kombination von α- und β^{E}-Ketten

Aufgrund der nur minimalen Ausprägung der genannten Veränderungen ist eine gesundheitliche Beeinträchtigung der betroffenen Individuen nicht zu erwarten Der Befund einer Hypochromie der Erythrozyten, die nicht durch einen Eisenmangel verursacht ist, sollte aber zum Anlass genommen werden, Hämoglobinanomalien und Thalassämiesyndrome in die differentialdiagnostischen Überlegungen mit einzubeziehen.

Zusammenfassung

Bericht über eine in Süddeutschland ansässige Familie, in der bei 10 Mitgliedern ein heterozygotes HbE (β26 Glu→Lys) nachgewiesen wurde. Die Identifizierung des anomalen Hämoglobins erfolgte durch Kombination verschiedener elektrophoretischer Techniken und mittels Fingerprintanalyse. Entgegen den Angaben in der Literatur bedingt Hämoglobin E auch im heterozygoten Status eine Hypochromie der Erythrozyten mit MCH Werten zwischen 23,6 und 26,2 pg. Ursächlich liegt der Hypochromie vermutlich eine verminderte Hämoglobinsynthese zugrunde

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Regulation of the β- and δ-Hemoglobin Genes

A Family with Hereditary Persistent Fetal Hemoglobin and β-Thalassemia

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Key Words. Hemoglobin genes · Hemoglobinopathies · Hereditary persistence of Hb F · Thalassemia

Abstract. We have studied a 41-year-old black male with the simultaneous occurrence of hereditary persistence of fetal hemoglobin (HPFH) and β-thalassemia, and his two postadolescent sons, each heterozygous for one of the traits. The son heterozygous for β-thalassemia had an elevated Hb A_2, but the index case did not. The data from this pedigree indicate that the δ-allele trans to the β-thalassemia gene was responsible for the increased δ-chain production. Evidence from other cases of combination HPFH and β-thalassemia indicates that regulation of the β- and δ-chain production in β-thalassemia is heterogeneous with respect to mechanism.

The chromosomal arrangement of the structural genes for hemoglobin chains has been delineated by genetic studies of patients simultaneously heterozygous for β- and δ-chain defects and in patients with the hemoglobin Lepore syndromes. These studies indicate that the β-, δ- and γ-loci are closely linked [14]. Furthermore, CEPPELLINI [4], NEEL [19] and MOTULSKY [17] have suggested that the β- and δ-hemoglobin genes are controlled by a single operator gene. However, the definitive proof of this has not been established due to the lack of a proper clinical model. The simultaneous occurrence of hereditary persistence of fetal hemoglobin (HPFH) and β-thalassemia in a patient, together with the independent segregation of genes coding for these traits, has allowed us to study the regulation of β- and δ-chain synthesis. Previously published reports of similar combined heterozygotes have been examined for the level of Hb A_2 synthesis in order to evaluate the regulation of δ-chain synthesis in β-thalassemia.

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Table I. Hematologic evaluation of the proband (A. B.) and two sons (R. B., K. B.)

	A. B.	R. B.	K. B.
Hemoglobin, g/dl	10 8	15 3	14.5
A, %	trace	NT	NT
A_2, %	1.8	—	5.4
F_A %	84.0	25.2	1 4
F_{Bm}, %	95 0	16 0	0 7
Hematocrit, %	38 0	47 4	48 0
Red blood cell count, 10^{12}/l	4 08	5 27	7 01
Mean corpuscular volume, μm^3	93	89	70
Mean corpuscular hemoglobin, pg	26	28	20
Mean corpuscular hemoglobin concentration, %	28	32	30
Reticulocyte count, %	14.3	1 3	2.1

By modified chromatographic procedure [7 10].
By alkali denaturation [2].
By Method of Schroeder *et al.* [21]
NT = Not tested.

Table II. Hemoglobin F characterization

Subjects	% F_{Bm}	Glycine	Alanine
A. B.	95.0	0.61	2.37
R. B.	16 0	0 34	2.74
K. B.	0.7	0.74	2.32
—			

By the method of Schroeder *et al.* [21].
By the method of Schroeder *et al.* [20].
—

heterozygote, or as a HPFH, β-thalassemia, or δβ-thalassemia homozygote.

Investigation of the proband's family disclosed that his brother and sister and his wife, the mother of his two sons, had normal values for Hb A, Hb A_2, and Hb F with normal erythrocyte morphology. His two sons had normal hemoglobin levels. One son K. B age 20 had 5 4 / Hb A_2 and 1.4% Hb F decreased MCV and target cells on peripheral smear. K. B. with 0 74 residues of glycine in the γCB-3 peptide falls into group II or the 'newborn group of β-thalassemia heterozygotes. Heterozygotes

Case Report

The proband, A. B a 41 year-old black male, was first seen at Charity Hospital in New Orleans (CHNO) as an outpatient in 1956 with refractory microcytic, hypochromic anemia The hemoglobin level varied between 10 and 11 g/dl. He was noted to have splenomegaly and chronic leg ulcers. He was not seen at CHNO again until 1967 when he was found to have severe folate deficiency anemia. The spleen had increased in size to the level of the umbilicus. After correction of the folate deficiency and 3 months after the last transfusion, the hemoglobin level was 10.2 g/dl and the ^{51}Cr red cell half life was 33 days. In 1968, splenectomy was performed at the time of cholecystectomy for symptomatic cholelithiasis. Postoperatively he has remained asymptomatic without overt jaundice, maintaining a hemoglobin level of 10–12 g/dl with folate supplements and a bilirubin of approximately 1.5 mg/dl.

Materials and Methods

Qualitative and quantitative hemoglobin analysis were performed by standard procedures. Qualitative hemoglobin analysis was determined by cellulose acetate electrophoresis at pH 8.7 in Tris-EDTA boric acid buffer at room temperature. Hemoglobin A and A_2 were quantitatively determined by DEAE-Sephadex chromatography [7 10] Hb F was determined semiquantitatively by alkali denaturation and is reported as $\%F_{A.D}$ [ ] The quantity of the Hb F was also estimated by a more accurate method which is based on the determination of the ratio of isoleucine to leucine and phenylalanine in the Hb F zone of each DEAE-Sephadex chromatogram [21] These data are presented as $\%F_{Ile}$. Heterogeneity of the γ-chain was determined by analysis of the purified γCB-3 peptide by the method of SCHROEDER *et al* [20] The distribution of Hb F in the red cells was determined by acid elution [15].

Results

Pedigree analysis of the hematologic findings of the proband and his two sons (table I II) and several members of the kindred was undertaken in order to establish the proband s genotype. The studies contained in this investigation were carried out 3 years or more after the proband had received his last blood transfusion His hemoglobin level was 10 8 g/dl with a trace (less than 1%) Hb A approximately 95% Hb F and 1.8% Hb A_2. Red cell anisocytosis and poikilocytosis, hypochromia target cells, and 100 000 nucleated red cells, µl were observed in the peripheral blood. According to HUISMAN *et al* [13] $G\gamma$ and $A\gamma$ composition of the Hb F (table II) would indicate that the proband should be classified either as a β-thalassemia and HPFH or as a $\delta\beta$-thalassemia and β thalassemia combined

led by HUISMAN *et al* [11] the cis δ-allele transcription was less than that of the trans allele.

The Negro form of HPFH is characterized by total absence of β- and δ-chain synthesis cis to HPFH gene with an associated compensatory increase in γ-gene activity [13 23] It is unknown whether this genetic condition is caused by an abnormal regulator or operator gene [4 17 19] or if it is due to a deletion of the closely linked β- and δ-genes [18] Whatever the cause, no cis β- or δ-gene activity takes place. Since the chromosome bearing the HPFH has a total absence of β- and δ-chain synthesis, any δ-chain production in the proband would have had to be due to the allele trans to the HPFH gene. Since he did not show an increase in Hb A_2 the δ-allele cis to the β-thalassemia gene must not be responsible for elevated δ-chain production in the heterozygous state. One may therefore, surmise that in the offspring heterozygous for β-thalassemia the allele trans to the β-thalassemia was responsible for the increased δ-chain production.

Of the 28 reported patients with combination HPFH and β-thalassemia, seven have had elevated levels of Hb A_2 [1 7 9 12, 14 22, 26] The variability of the Hb A_2 levels of individuals simultaneously heterozygous for β-thalassemia and δ-chain variants [3, 11] and HPFH and β-thalassemia indicates that the regulation of the β- and δ-gene transcription in β-thalassemia gene is heterogeneous with respect to mechanism. The analysis of the hematologic findings in this family indicates that the adjacent structural β- and δ-genes constitute a coordinated unit of transcription as has been previously proposed [4 17 19]

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of group II have glycine values of approximately 0.7 as in Hb F of the newborn whereas, those of group I have glycine values of approximately 0.4 as in the traces of Hb F in the adult [13]. The other son, R.B. age 18, had 2.2% Hb A_2, 25.5% Hb F and normal MCV, MCH and erythrocyte morphology. The 0.34 glycyl residues in the γCB-3 peptide does not distinguish between HPFH and δβ-thalassemia. The value of 0.4±0.1 residues has been found for both δβ-thalassemia and HPFH traits [13]. The F_{II} of 16% is rather low for HPFH heterozygotes and is more suggestive of δβ-thalassemia trait [13]. However, because of the normal erythrocyte morphology and because the Hb F was homogeneously distributed among the erythrocytes, the findings were most consistent with a diagnosis of heterozygous HPFH of the Negro type [6, 8, 12, 22, 23]. These hematological studies, therefore, indicated that the proband was simultaneously heterozygous for β-thalassemia and HPFH.

Discussion

The combination of genes responsible for the HPFH and β-thalassemia was characterized in the proband by elevated levels of Hb F, low levels of Hb A_2, and almost complete absence of Hb A. Both thalassemia and HPFH are very heterogeneous [24]; however, previous reports of individuals with this combination of genes have had either complete absence or moderate amounts of Hb A [9]. In contrast, the proband had less than 1% Hb A. The presence of this unusual amount of Hb A may be due to minimal transcription of the β-gene in the nucleated red cells [5] which were present in large numbers in the peripheral blood.

Individuals homozygous for β-thalassemia have decreased δ-chain synthesis [24], and β-thalassemia heterozygotes typically have elevated Hb A_2 levels [16]. This indicates some type of regulatory mechanism. CEPPELLINI [3] and HUISMAN *et al.* [11] postulated that the increase in the δ-chain synthesis in β-thalassemia heterozygotes is due to both the cis and trans alleles. Moreover, this would indicate that the regulation is due to a diffusible substance. In both studies, evidence for the dual increase in gene activity was derived from individuals simultaneously heterozygous for β-thalassemia and a δ-chain variant, Hb B_2. The affected individuals had variable levels of normal and variant hemoglobin components. Both cases studied by CEPPELLINI [3] showed greater transcription by the δ-allele cis to the β-thalassemia gene. However, in 70% of the 14 cases stud-

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the present paper The drug chosen – methotrexate (Mtx) – is widely used in the management of leukaemia. Of greater importance for our purposes is the fact that it acts solely on the proliferating compartment. The perturbation of the cell cycle induced by Mtx on leukaemic blasts results in what has been described as a 'free, normal-entry-no-exit model' i.e. the entry of cells into the S phase is not impeded. Once there, they are blocked and die in the compartment (cytolytic effect) [5] However, the doses employed at present do not prevent the progress, albeit postponed (cytostatic effect only), of a very small cell fraction of Mtx-stricken cells to mitosis [5 8] Furthermore, cells subjected to this purely cytostatic effect are bearers of a biochemical injury that may interfere with their ability to pass through the next mitotic cycle [1]

Materials and Methods

Blasts labelled *in vivo* with ^{3}H-thymidine (^{3}H TdR) were followed during and after brief Mtx management in two previously untreated cases of acute lymphoblastic leukaemia (ALL). In case 1, the fraction of cells in the S phase was first subjected to Mtx, after which the fate of cells blocked in S and those subsequently entering this phase from G was followed. In case 2, only those blasts that were in S at the time when the drug was administered were studied.

Case 1 G A., a 20-year-old male presented high bone marrow cellularity (400,000 cells/µl) with 90% lymphoblasts, as shown by negative Sudan black and peroxidase reactions and PAS positivity The peripheral white cell count was 11,300/µl (94% lymphoblasts). Two doses of 0.5 mg/kg Mtx were given at an interval of 12 h, followed by 10 mCi ^{3}H TdR (specific activity 10 Ci/mM) after four 1 h. Marrow samples were taken 1, 10, 20, 48 h, 5 and 8 days later Marrow and peripheral blood cellularity fell rapidly and the marrow blasts were 2% on the 8th day Later complete remission lasting 6 months was obtained with conventional protocol.

Case 2. P G 30-year-old male presented an even more striking picture: virtually complete marrow metaplasia (98% lymphoblasts, as shown by cytochemical reactions), high bone marrow cellularity (1,400,000/µl), and peripheral white cell count of 3,000/µl (30% lymphoblasts). 0.5 mg/kg Mtx and 10 mCi ^{3}H TdR (specific activity 10 Ci/mM) were given simultaneously Marrow samples were taken at the same intervals as in case 1 and on the 12th day The early picture showed drastic reduction of both marrow and peripheral blasts. By the 12th day however slight increase in marrow blasts was apparent and chemotherapy was started again.

Autoradiography Smears from each marrow blood sampl were fixed in Carnoy and covered with Ilford K_2-emulsion. After exposure at 4 °C for 191 days (case 1) or 290 days (case 2), the slides were developed, fixed and stained with

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Persisting Production of Out-of-Cycle Blasts during Methotrexate Therapy in Acute Leukaemia[1]

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Key Words Autoradiography Cell kinetics in leukaemia Leukaemia therapy Lymphoblastic leukaemia Methotrexate Microspectrophotometry

Abstract Kinetics of the blast populations in two cases of acute lymphoblastic leukaemia during treatment with methotrexate have been studied *in vivo*. Beside a striking cytocidal effect on blasts arrested in the S phase and a synchronization and a slowing of those in the G_1 phase, the most important finding was the persistence of a flux of drug-affected cells from the proliferation to the non-proliferating compartment. Some of these non-proliferating cells were able to re-enter subsequently the mitotic cycle. This fact can explain why a cell-cycle-specific drug fails to eradicate completely acute leukaemic cells in man.

A pool of out-of-cycle leukaemic cells (G_0) relatively insensitive to drugs and, as stem cells, able to replenish the proliferating compartment is the major kinetic obstacle to the eradication of leukaemia in man [6, 12, 14] Cure can only be assumed when all leukaemic precursors, including non-proliferating blasts, have been destroyed. This has been theoretically envisaged via drug management designed to induce a complete drain of cells from the G_0 compartment [7 10 13] However it is obvious that such a plan would be baulked by a feedback of cells from the proliferating to the G_0 compartment during a treatment of this kind, since the consequent self maintenance of the latter compartment will guarantee the supply of leukaemic stem cells.

A flow of cells from the proliferating to the G_0 compartment during cycle-specific chemotherapy has been assumed though not yet proved [7] The production of G_0 cells during such treatment, is investigated in

[1] Supported by CNR, Roma.

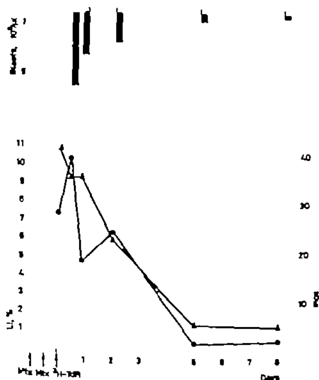


Fig. 1 Bone marrow LI (●), MGC (▲) and blasts per μl after Mtx and ^{3}H-TdR pulse in case 1.

compartment. Had they been vital (i.e. mostly in G_0), their LI should have remained constant during this time since no more labelled large cells appeared.

A total kill of small labelled blasts was not achieved, however since a few were still detected on the 8th day. The kinetic picture in the first 10 h suggests that Mtx also slows down the G_2 phase to give partial cell synchronisation. The presence of a mostly non-labelled mitosis peak at the 10th hour points to synchronisation, detectable in M, of cells that were for the most part out of S when the tracer was given.

Division of part of the proliferation pool in spite of Mtx was also clear when the DNA content of labelled blasts at 1 and 20 h was compared (fig. 3). All values were over 2 DNA at 1 h, whereas diploid con-

Giemsa buffered to pH 7.2. The following parameters were determined: number of marrow and peripheral blasts, mitotic index (MI), percentage of labelled blasts (LI) on at least 2,000 cells, labelled mitosis percentage on at least 100 figures, and mean grain count (MGC) on at least 200 cells. The population was divided into large and small blasts according to the diameter of at least 1,000 cells; LI and MGC were determined for these subpopulations.

Microspectrophotometry (case 1) A photographic map was used to identify 100 labelled blasts and their DNA content was determined at 1 h and 20 h. For this purpose, the autoradiographic grains were removed with potassium ferricyanide and the emulsion was digested with 0.1 M trypsin [4 11]. After Feulgen staining, DNA values were determined in 5,400 monochromatic light with a Barr & Stroud GN 2 spectrophotometer The values of absorbance were expressed in arbitrary units.

Results

Case 1 (Mtx 24 and 12 h prior to ^{3}H TdR) Marrow blast values were unchanged when the tracer was injected Thereafter there was a slight reduction at the 10th hour followed by a rapid fall to very low levels on the 8th day heralding the complete haematological remission (fig. 1). There was an initial rise in LI and a slight fall in MGC, pointing to the division of a cohort of blasts at the end of the S phase and unaffected by the drug when the tracer was given. Later a drastic fall in LI (10th hour to 5th day) was the result of the cytocidal effect of the drug. The persistence of a certain degree of proliferation, however may be deduced from figure 1 The MGC was almost halved at the 48th hour and on the 5th day while an LI spike was also noted at the 48th hour

More detailed evidence of proliferation was obtained on examination of the mitotic activity (MI and percent of labelled mitoses) and by a separate study of the behaviour of the large and small blast compartments. Figure 2 shows that cells in mitosis were still present for the first 48 h Between 10 and 48 h labelled mitoses were observed, pointing to the only partly cytocidal effect of Mtx. After the 10th hour a sharp reduction in the LI of the large blasts was accompanied by the emergence of labelled small blasts, whose percent value reached its peak at the 48th hour On the 5th and 8th days, no more labelled large blasts were seen, the percentage of labelled small blasts fell and stayed low and the MI was 0 This means that little or no replenishment of the small by the large blast compartment took place between the 2nd and the 5th day The fact that small blast LI decreased strongly between the 2nd and the 5th day shows that most of these small cells were probably within the Z

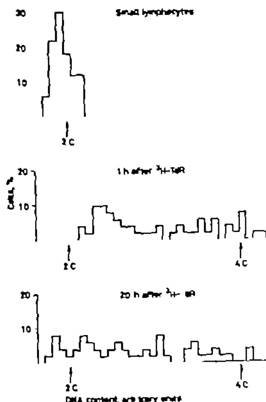


Fig. 3 DNA content (arbitrary units) of *in vivo* labelled blasts after Mtx in case I.

ways present. The MI fell during the first 48 h and then rose considerably until the 5th day. Thereafter the values fell again, though no change was noted between the 8th and the 12th days. It can also be seen that a labelled mitosis fraction was always present. The fact that this fraction was very low (11.5%) at the 10th hour i.e. when a value of 100% would have been expected, points to abnormal protraction of the G phase, as in case 1. The presence of labelled mitoses soon after the administration of Mtx and ³H TdR shows that the drug is also cytostatic for some cells in the S phase. The labelled mitoses observed towards the end of the experiment, on the other hand, can hardly be explained by a prolonged cytostatic effect. Comparison of the large and small blast LI

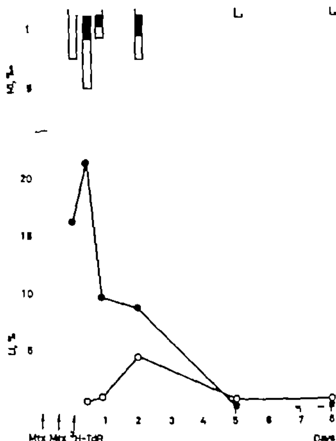


Fig 2 Bone marrow LI of large (●) and small (O) blasts, MI (□) and fraction of labelled mitoses (■) after Mtx and ³H TdR pulse *in vivo* in case I

tents were found in about 15–20% of cells at 20 h. Thus shows both a block or abnormal lengthening of the S phase and the ability of part of the proliferating fraction to overcome this drug-induced block and divide.

Case 2 (Mtx and ³H TdR given together) Mtx proved rapidly cytocidal. There was a sharp fall in marrow blasts in the first 48 h with a further decrease to a nadir on the 5th day followed by a rise on the 8th and 12th days (fig. 4) During the first 48 h there was a decrease in LI of the total cell population, whereas the value rose on the 5th day and remained virtually the same till the end of the experiment. The MGC fell rapidly during the first 22 h and then more slowly to reach the halving point by the 5th day Figure 5 shows that mitotic activity was al

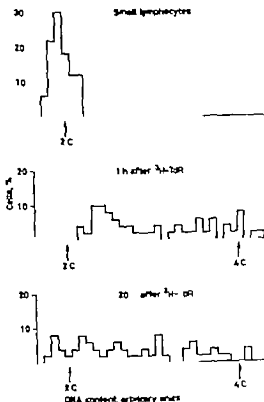


Fig 3 DNA content (arbitrary units) of *in vivo* labelled blasts after Mtx in case L

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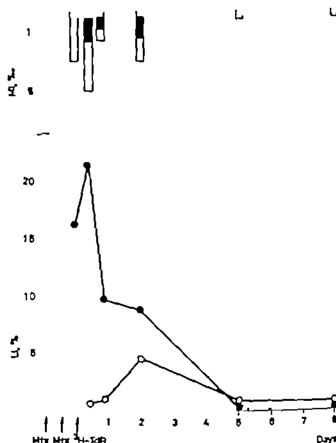


Fig 2 Bone marrow LI of large (●) and small (O) blasts, MI (□) and fraction of labelled mitoses (■) after Mtx and ³H TdR pulse *in vivo* in case 1

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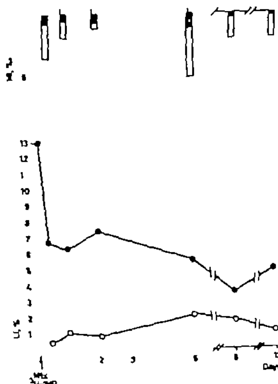


Fig. 5. Bone marrow LI of large (●) and small (O) blasts, MI (□) and fraction of labelled mitoses (■) after Mtx and ³H-TdR pulse *in vivo* in case 2.

Here the pattern is different from that in case 1, where the prior administration of Mtx probably rendered later recruitment less evident, so that the small blasts could only have been in the Z compartment.

Discussion

Our results enable us to draw a number of conclusions concerning the short- and long-term effects of Mtx on the proliferation kinetics of leukaemic blasts. It is thought that the drug's mechanism is essentially directed to the irreversible arrest of cells in the S phase, whereas the passage from G_1 to S is left free and proliferating cells in G itself are

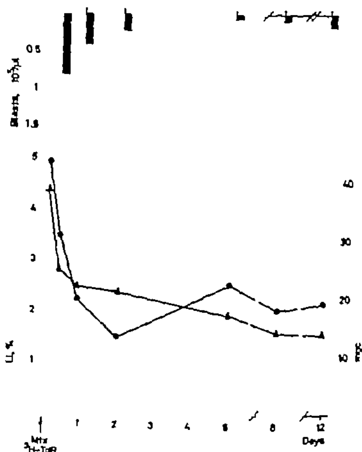


Fig. 4. Bone marrow LI (●), MGC (▲) and blasts per µl after Mtx and ³H TdR pulse in case 2.

offers further evidence of the simultaneously cytolytic and cytostatic effect of Mtx.

Moreover the interdependence of the two compartments could be followed throughout the experiment. During the first 22 h, there was a rapid marked fall in large cell LI. A cohort of labelled small blasts was still produced however showing the persistence of proliferative activity confirmed by the presence of labelled mitoses. The fall in large blast LI was less sharp from the 2nd to 8th days and there was a marked increase in the percentage of labelled small blasts. Here the continuous cytocidal effect of Mtx was being noticeably offset by proliferating cells proceeding to mitosis after a protracted S phase. In the final stage (8th to 12th day) the LI of large and small blast rose and fell, respectively

as long as 5 days without damaging their ability to divide. Moreover if synchronization is involved, the percentage of labelled mitoses should be high, whereas no more than 19% of the figures were labelled. Therefore, recruitment as an overture to subsequent expansion of the leukaemic population would seem more in keeping with the kinetic data. The failure of the proliferating compartment LI to fall is compatible with the return of small blasts in G into the cycle, some of these being labelled because they were generated by the mitotic activity that persisted during and immediately after (10–48 h) administration of Mtx.

The observation of a small number of labelled mitoses on the 5th day can be explained by assuming that some recruited cells were already in the process of dividing at the time of the experiment. Recruitment of cells, including some derived from a P-to-G_o flow during the action of Mtx is borne out by the final data. Between the 8th and 12th days, when the blast expansion is under way large blast LI values increase while small blast LI decrease. Since Mtx and the tracer were given together at the start of the experiment, the increase in LI on the 12th day was only possible at the expense of labelled cells that had by then passed into the non-proliferating compartment. In other words, a return of small blasts into the proliferating compartment is the only possible explanation for such an increase. Positive corroboration of this can be seen in the presence of labelled mitoses on the 12th day While it is just possible that those observed on the 5th day may be divisions of cells that have resisted a prolonged Mtx-induced block in S, it can hardly be supposed that protraction of the block for a further week would be compatible with cell survival and eventual mitosis [1]

The findings in the second case, therefore, are in line with those in the first, namely that some Mtx-attacked cells manage to proceed to mitosis and so form a pool of small non-proliferating blasts (P-to-G flux) It is also clear that part at least of this pool belongs to the G compartment since some of its cells can recommence their proliferative activity after a long period of mitotic rest. These quiescent (G_o) blasts, in fact, conserve their stem character and are thus responsible for the expansion of the leukaemia population.

References

1 Borsa, J Cytotoxic mechanism of folate antagonists. Ann. N.Y Acad. Sci. 186: 359–362 (1971).

relatively unaffected [3 5 9] Our data, on the other hand, particularly those showing a rapid fall in cellularity and the apparent disappearance of the proliferating compartment in case 1 indicate that the G_1 phase may also be sensitive to Mtx. In this connexion, it will be recalled that BORSA and WHITMORE [2] have shown the existence of a stable bond between Mtx and folicoreductase in G_1 cells that are immediately destroyed on entering the S phase. Our observation of a high percentage of unlabelled mitoses 10 h after the tracer in both cases also suggests that the effects of Mtx are also felt by blasts in G_2 this, in fact, is abnormally long.

The persistence of mitoses, during the time when damage to cells in S is at its maximum and the appearance of labelled mitoses (with maximum percent values at 20 and 48 h) shows that Mtx also has a merely cytostatic effect on certain cells whose progress to mitosis is delayed, not stopped Evaluation of DNA content in case 1 showed that these mitoses were not spurious, i e. that they gave rise to a clearly identifiable population of cells in Z or G_0. The fate of this small cohort of labelled blast cells is a pointer to their part in the leukaemic population.

In case 1 progressive destruction to the point of apparent disappearance of blast cells was observed with no sign of the return of proliferative activity Labelled cells belonging solely to the non-proliferating fraction only were noted for as long as it was possible to detect leukaemic cells (5th and 8th days) This shows that in spite of treatment, there is a continuous flow of large non proliferating blasts into the temporarily or permanently non proliferating small blast compartment. In case 2, the massive cytocidal effect was followed by re-expansion of the leukaemic population by the 8th and 12th days. On the 5th day the marrow and peripheral blasts reached their lowest values.

The kinetic data were interesting Small blast LI which, as in case 1 began to rise 10 h after ^{3}H TdR, now reached its peak, whereas, by contrast with case 1 the large blast value was also at a relatively high value. The MI after a distinct and progressive fall returned to approximately its start level. Two explanations can be offered of the 5th-day kinetic picture (1) a cohort of blasts suffered no more than the cytostatic effect of Mtx overcame the block in S and passed through G_2 to reach mitosis at the same time (synchronization) (2) massive destruction caused by the drug provoked a recall of blasts from the G_0 compartment (recruitment) The first explanation can be rejected on the ground that it would mean that Mtx is capable of holding up a number of cells in S for

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Evaluation d'une méthode d'étude des agrégats plaquettaires par fixation au formol[1]

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Key Words. Aggregation index Platelet aggregation Thrombocytes

Abstract. An approach for testing platelet aggregates by formol fixation is presented. The investigations are made either *in vitro* by the study of aggregation in the presence of ADP or *in vivo* after thrombin perfusion in the rabbit. The calculation of an 'index of aggregability' comparing fixed and non-fixed platelets permits an evaluation of the presence of aggregates.

La mise en évidence des agrégats cellulaires dans le sang a fait l'objet de techniques très diverses. En ce qui concerne les plaquettes, la technique la plus utilisée *in vitro* est certainement la technique néphélométrique [1 2, 6]. Cependant, cette technique qui étudie surtout l'aptitude des plaquettes à s'agréger ne permet pas d'apprécier les agrégats circulants ou présents dans le sang total. Une technique plus spécifique décrite initialement par Swank et Fellman [7] et Swank *et al.* [8] pour le sang total, consiste à étudier la différence de pression induite par le passage à débit constant d'une suspension à travers des filtres calibrés [5]. La présence d'agrégats entraîne une augmentation de la perte de charge à travers le filtre par colmatage. Une modification de cette technique a été proposée par Hornstra [3 4] Elle permet des études directes sur du sang natif. Mais ces techniques, bien qu'intéressantes, ne permettent pas une appréciation des agrégats *in vivo*

Le but de ce travail est d'essayer d'évaluer l'intérêt et de préciser les limites d'une technique décrite récemment par Wu et Hoak [9], et qui

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Evaluation d'une méthode d étude des agrégats plaquettaires par fixation au formol[1]

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Abstract. An approach for testing platelet aggregates by formol fixation is presented. The investigations are made either *in vitro* by the study of aggregation in the presence of ADP or *in vivo* after thrombin perfusion in the rabbit. The calculation of an 'index of aggregability' comparing fixed and non-fixed platelets permits an evaluation of the presence of aggregates.

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consiste à fixer les agrégats dès le prélèvement par un mélange EDTA formol.

Matériel et Méthode

Principe de la méthode Elle consiste à définir un index d'agrégation, comme étant le rapport entre les numérations plaquettaires de deux prélèvements, l'un avec comme anticoagulant un mélange EDTA-formol et le second de l'EDTA seul. Dans le premier prélèvement, les agrégats plaquettaires sont «fixés» par le formol et disparaîtront lors de la préparation du PRP alors que dans le second, les agrégats seront dissociés et par suite, la numération plus élevée. Le rapport entre les deux valeurs de la numération définit ce que l'on peut appeler «l'index d'agrégation Plus celui-ci sera bas, plus le sang initial contiendra d'agrégats plaquettaires.

Matériel. Deux prélèvements de sang de 4 ml sont réalisés dans des tubes contenant soit un mélange EDTA formol avec 1% de formol, soit de l'EDTA seul. La solution anticoagulante de base est l'EDTA 0 077 M ajustée à pH 7 par du phosphate disodique en poudre. La solution EDTA formol est préparée à partir de 7,5 ml d'EDTA et 2,5 ml de formol à 4%. Les premières expériences nous ont montré que le mélange EDTA-formol doit être réalisé peu de temps avant le prélèvement. Une fois prélevé le sang est alors incubé 15–20 min à la température ambiante. Les PRP sur lesquels seront réalisés les numérations sont alors préparés par centrifugation lente à 20 °C.

Méthode expérimentale Afin de tester la validité de la méthode, deux types d'expériences ont été réalisées. La première, *in vitro* consiste à étudier la formation d'agrégats plaquettaires par adjonction de concentrations variables d'ADP à un PRP prélevé sur citrate (1:9) puis fixé par le mélange EDTA formol. La seconde est *in vivo* chez le lapin, par perfusion de thrombine à une dose variant de 0,5 à 2 unités par kilogramme de poids. Dans ce dernier cas, les prélèvements ont généralement été réalisés 15 min après la fin de la perfusion.

Résultats

Résultats in vitro Nos résultats expérimentaux *in vitro* sont résumés sur le tableau I Ils montrent que, pour des concentrations d'ADP variant de 0 1 à 10 μ M/ml l'index d agrégation est bas alors que pour des valeurs de la concentration d ADP plus faibles (inférieure à 10^{-2} μ M/ml) ce rapport se rapproche de la valeur normale unitaire.

Résultats in vivo Après avoir essayé des doses variant de 0,5 à 2 unités/kg, nous nous sommes arrêtés à une dose moyenne de 1 unité/kg perfusée en 10–20 min. Le tableau II montre que, dans les 8 expériences réalisées, l'index d agrégation a toujours été diminué d'environ 50% par rapport à la valeur normale initiale avant la perfusion de thrombine.

Tableau I. Variation de l'index d'agrégation *in vitro* après adjonction d'ADP

ADP µmol	Valeur moyenne	Valeurs extrêmes	Nombre de mesures
Témoin	1,01	0,81 1 18	9
10^{-8}	0,91	0,75–1,03	6
10^{-7}	0,84	0,69–0,95	7
10^{-6}	0,65	0,45–0,79	6
1	0,33	0,15–0,43	6
5	0,18	0,11–0,23	4
10	0,14	0,07–0,30	7

Tableau II. Variation de l'index d'agrégation chez le lapin après perfusion de 1 U/kg de thrombine

Expérience No.	Index d'agrégation avant perfusion	après perfusion
1	1,06	0,38
2	0,92	0,48
3	0,88	0,60
4	0,92	0,38
5	0,83	0,50
6	0,92	0,48
7	0,93	0,49
8	0,91	0,54
Moyenne	0,92	0,48

Conclusion

Cette étude nous a permis de démontrer la validité d'une nouvelle technique de mise en évidence des agrégats plaquettaires. Cette méthode est simple et de mise en ceuvre facile même au lit du malade à condition de disposer d'une centrifugeuse de paillasse permettant de séparer les PRP après fixation ce qui évite une hémolyse trop importante. La reproductibilité de la méthode est assez bonne avec cependant, des variations de l'ordre de 10–15% autour de la valeur normale.

Résumé

L'auteur teste une nouvelle méthode d'approche des agrégats plaquettaires par fixation au formol. Les études sont réalisées soit *in vitro* par étude de l'agrégation à l'ADP soit *in vivo* chez l'animal. Les possibilités en clinique sont envisagées.

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Platelet Aggregation and Adhesiveness in Classical Factor X Deficiency and in the Abnormal Factor X (Factor X Friuli) Coagulation Disorder[1]

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Key Words. Blood coagulation · Coagulation disorders · Factor X deficiency · Factor X Friuli · Platelet adhesiveness · Platelet aggregation

Abstract. Platelet aggregation to common inductors and to Ristocetin, Thrombofax and Inosithore is normal in congenital factor X deficiency and in factor X Friuli coagulation disorder. Washed normal platelets resuspended in the patient' plasma and in adsorbed normal plasma showed a normal aggregation. On the contrary normal platelets resuspended in normal serum failed to aggregate. These studies indicate that factor X plays no role in normal platelet aggregation.

The protein requirements for a normal platelet aggregation are still ill-defined [2, 17]. Still poorly understood is the interaction between the only two factors, anti-von Willebrand factor and fibrinogen, which seem to play a major role in normal platelet aggregation and the platelet membrane. However, it is known that platelet aggregation to common inductors is variably defective in congenital afibrinogenemia and that in classical von Willebrand disease platelet aggregation to Ristocetin is absent [6, 12, 14, 21].

The claims concerning the role played by other factors appear to be less justified. Bang *et al.* [2] have dealt with factor VIII, IX and XI deficient plasmas and found that the activities associated with these factors played no role in platelet aggregation. Several data have been presented indicating that factor XII either inhibits or stimulates platelet aggregation [1, 7]. In a patient with Hageman trait a concomitant and distinct defec

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tive second phase platelet aggregation was described [16] We have demonstrated recently that in congenital factor XII deficiency platelet aggregation is normal and that ellagic acid a potent factor XII activator is unable to affect platelet aggregation both in normal and in factor XII deficient subjects [9] Recently it was postulated that the prothrombin complex may play a role in platelet aggregation [15]

The key factor of the prothrombin complex is surely factor X since this factor is uniquely involved both in the intrinsic and in the extrinsic system. We had the unusual opportunity during the past years to study a patient with severe factor X deficiency and several patients with the factor X Friuli abnormality [4 5 7 8] This is the first paper dealing with platelet aggregation in factor X deficient or abnormal factor X plasmas.

Material and Methods

Material and methods have been discussed in detail elsewhere, only new data will be given herein. Factor X deficient plasma was obtained from a patient previously reported [8] Abnormal factor X (factor X Friuli) plasma was obtained from a patient previously reported [5] The main features of these two patients are summarized in table I. Bleeding time, platelet count and clot retraction in these patients were normal.

Ionophore A 23187 was supplied by Dr R. L. Hamill, Eli Lilly Laboratory Indianapolis, Ind. Ionophore was dissolved in ethanol and added to the platelet samples (final concentration of ethanol 0 1 v/v) at a final concentration of 0.4 M. Ristocetin sulfate was supplied by Lundbek Co Copenhagen, Denmark. ADP adrenaline and collagen were supplied by Stago Laboratories, Asniéres, France. Thrombofax was supplied by Ortho Laboratories, Raritan NJ

Adsorbed normal plasma was obtained by mixing 2 ml of citrated plasma with 200 mg of borico sulfate (Bayer) for 5 min. The supernatant was then separated by centrifugation at about 1 000 r for 5 min and the entire procedure repeated once again. After this double adsorption the plasma did not clot upon addition of calcium and tissue thromboplastin (prothrombin time greater than 300 sec partial thromboplastin time greater than 500 sec).

Normal serum was obtained 2 h after clotting of normal blood in a glass tube. The serum was separated by centrifugation and incubated 10 min at 37 °C and subsequently kept at room temperature.

Platelet aggregation in PRP was carried out as previously reported using ADP (0.5 μg/ml) adrenaline (65 μg/ml), collagen (40 μg/ml) Ristocetin (1.5 mg/ml) and Thrombofax (0.2 ml for 0.8 ml of PRP) [6].

Platelet adhesiveness was carried out according to a modification of the method proposed by Bowie *et al.* [3] and Hellem [11]. 5 ml of native blood were drawn into a plastic syringe 1 ml was inserted into a plastic tube containing 7.5 mg EDTA. The syringe was then placed in a syringe pump apparatus (Mascia Brunelli, Milan,

Table I. Main laboratory features of the two patients investigated

	PTT sec	PT sec	Factor X level, % tissue thromboplastin	Russell's viper venom + cephalin	Antigen (protein)
Classical factor X deficiency	144	125	<0.1	<0.1	<1
Factor X Friuli disorder	66.7	33	7[1]	92	110
Normal values	32–42	14	85–120	85–120	60–160

[1] Average of values obtained using whole and partial thromboplastin.

Italy) and glass bead column was attached to the knob. This operation required less than 30 sec. The column contained 1.45 g of glass beads and was 14 cm long. The diameter of the glass beads was 0.3 mm. The flow rate of the blood was 4 ml/min. The effluent blood was collected in plastic tubes containing 7.5 mg EDTA. The platelet count for the percentage of platelet retention was carried out in the 3rd ml of the effluent blood.

Washed platelets were obtained by the method of WALSH [19] with albumin density gradient separation; the separation was repeated four times. The test system consisted of 0.5 ml of final washed platelet suspension (600,000/µl), 0.5 ml of normal or test plasma and 0.1 ml of the following aggregating agents: collagen 40 µg/ml, Ristocetin 1.5 mg/ml, Ionophore 0.4 µM, ADP 3 µg/ml.

Results

The results are summarized in table II. Platelet aggregation, regardless of the aggregating agent, was normal both in congenital factor X deficiency and in factor X Friuli coagulation disorder. The resuspension studies in patient's plasma or in adsorbed normal plasma using washed normal platelets showed also a normal pattern in every instance. On the contrary no aggregation was noted when normal platelets were resuspended in normal serum. Platelet adhesiveness to glass beads was normal in both cases.

Discussion

Platelet aggregation to all known inductors is normal in congenital factor X deficiency. Similar results were obtained in the factor X Friuli coa-

Table II Platelet aggregation indexes and platelet adhesiveness in classical factor X deficiency and in factor X Friuli disorder

	Platelet aggregation							Platelet adhesiveness, %
	ADP MA	adrenaline (56 µg) MA	collagen (40 µg) MA	LPh	Thrombofax (0.2 ml) MA	Ionophore (0.4 µM) MA	Ristocetin (1.5 mg) MA	
Classical factor X deficiency	69[1]	75	60	60	7?	70	87	79
Factor X Friuli disorder	77[1]	70	77.5	50	71	80	81	80
Normal values of PRP	67 ± 18.2[1]	68 ± 11	63 ± 19 4	90 ± 38	70 ± 12	72 ± 16	74 ± 12.5	82 ± 9
Washed normal platelets in factor X deficient plasma	66[2]	–	55	100	–	69	60	
Washed normal platelets in factor X Friuli plasma	60[2]	–	61	60	–	60.5	74	
Washed normal platelets in normal plasma	60 ± 13 1[1]	–	62 ± 17	98 ± 26	–	73 ± 10.3	71 ± 14 8	

MA = Maximal amplitude of the curve, % LPh = lag phase, sec.
[1] 0.5 µg ADP
[2] 3 µg ADP

gulation disorder Platelet adhesiveness was also normal in both conditions.

Our patient with classical factor X deficiency had a factor X level of less than 0 1% and no detectable factor X protein was present in immunological assays. On the contrary the patient with factor X Friuli abnormality had a factor X antigen of about 110% of normal and about 7% factor X activity using a tissue whole or partial thromboplastin [5 7] It may therefore, be concluded that neither factor X activity nor factor X antigen are necessary for platelet aggregation and adhesiveness. It is well

known, on the contrary that platelet phospholipid play a fundamental role in the generation of activated factor X [20]

The possibility that the 'lack' of factor X antigen or activity may be compensated, under certain circumstances, by other plasmatic compounds may not be completely ruled out but seems unlikely The resuspension studies confirmed fully these negative findings. Normal platelet resuspended in the patient's plasma showed a normal aggregation pattern. This indicates that no platelet aggregation inhibitor was present in the patient's plasma and again that factor X antigen and/or activity was not necessary for platelet aggregation. The fact that a normal aggregation was obtained even after resuspension in adsorbed normal plasma seems to indicate that none of the prothrombin complex factors is important for normal platelet aggregation. The prothrombin time and the partial thromboplastin time of the adsorbed plasma was greater than 300 and 500 sec, respectively and, therefore, it can be maintained to be completely free of factor II VII, IX and X. The possibility that platelet suspension contained traces of factor X may be safely ruled out. The addition of such platelet preparation to the patient's plasma failed in fact to shorten, even for a few seconds, the basal prothrombin time.

Our negative studies are not in contradiction with the data presented by MIALE and KENT [15] who have shown an inhibitory effect of platelet aggregation on the part of an anti-prothrombin complex factor's antiserum. The addition of an antiserum, namely of a protein complex, to an aggregation medium cannot be maintained to indicate surely an inhibition due to coagulation factors 'blockage' or 'neutralization' by the antiserum. The antiserum surely contains several proteins which may exercise an effect on platelet aggregation itself. We have observed for example that an antifactor VIII antiserum added to the cuvette is capable of inhibiting sharply platelet aggregation not only to Ristocetin, which could be expected, but also to ADP

Furthermore similar results were obtained using an anti-prothrombin antiserum and an anti-γ antiserum indicating, probably the nonspecificity of the phenomenon. However the possibility that anti-coagulation factors' antisera may play a certain specific role in platelet aggregation inhibition needs further studies before any final conclusion is drawn.

Finally our negative results are also not in contrast to those studies which have shown an aggregating capability of activated factor X preparations. In these cases the aggregating activity of the preparations is in fact probably related to the presence of traces of thrombin. These traces

of thrombin may be either present as such in the factor Xa preparation or may become apparent as a result of the action of factor Xa on traces of prothrombin remaining on the platelet membrane or still present in the platelet suspension [13 18 20]

Since preliminary results in our laboratory indicate that platelet aggregation is also normal in factor II VII and IX congenital deficiencies and in view of the resuspension studies in adsorbed normal plasma, it may be concluded that the prothrombin complex factors do not seem to play a significant role in platelet aggregation and platelet adhesiveness to glass.

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Table I. Platelet aggregation in patients with congenital F XIII deficiency by four aggregating agents

Patients	Age years	Sex	ADP (10^{-5}M/ml)	Epinephrine (5 μg/ml)	Collagen (2%)	Ristocetin (2 mg/ml)
R.T.	4	M	N	N	n.p.	n.p.
H.Q.	6	M	N	N	N	N
H.G.	9	F	N	N	N	N
S.A.	5	F	N	N	n.p.	p.

N = Normal n.p. = not performed.

activity was found to be less than 1 / in all cases, by the method of Bochmann and Attar [4].

Platelet-rich plasma (PRP) was prepared from venous blood, which was anticoagulated with 1.10 volume of 3.8% sodium citrate in plastic tube, by centrifugation at room temperature for 20 min at 600 rpm. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP for 10 min t 15,000 rpm. Platelet counts were performed by phase contrast microscopy using the Brecher-Cronkite method [3]. Platelet counts in the PRP samples were adjusted to 400,000/μl with autologous PPP.

Platelet aggregation was studied spectrophotometrically using platelet aggregometer (Chrono-Log Corp Broomall, Pa.) and changes in light transmission were recorded continuously on model 702 strip chart recorder. Agents used to induce platelet aggregation were adenosine-5-diphosphate (ADP), epinephrine, collagen and ristocetin. ADP (Sigma, St. Louis, Mo.) was dissolved in Tyrode's solution to the required concentrations (10^{-5} M/ml). Aqueous epinephrine and ristocetin were diluted with saline in concentrations of 5 μm/ml and 18 mg/ml, respectively. Collagen suspension (Sigma) was prepared as previously described [19].

Results and Comments

The primary and secondary aggregation values of the platelets with ADP and epinephrine in the four patients, and collagen- and ristocetin-induced platelet aggregation in two patients was studied, and no abnormality could be shown (table I).

Hemorrhagic diathesis due to congenital FSF deficiency was first described by Duckert *et al.* [6] in 1960, and more than 65 cases have been reported since then [14]. With this deficiency patients have a dis-

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Platelet Aggregation in Congenital Factor XIII Deficiency

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Key Words Blood coagulation Factor XIII deficiency Platelet aggregation

Abstract. Platelet aggregation has been studied in patients with congenital factor XIII deficiency By using epinephrine, ADP ristocetin and collagen it was shown that platelet aggregation is normal.

Plasma factor XIII (F XIII fibrin stabilizing factor FSF) is present as a proenzyme in the plasma and is converted to an enzymatically active form by thrombin Platelet FSF is also present in the proenzyme form and activated by thrombin [13] Although plasma and platelet FSF activities are similar on fibrin monomers [20] physicochemical properties, including molecular weights, carbohydrate and amino acid compositions of them are different [3 8] By immunochemical studies it is shown that plasma F XIII has two components one of which is similar to platelets FSF [10]

Several parameters of coagulation in the patients with congenital F XIII deficiency have been studied, but we could not find any study related to platelet aggregation The purpose of the present study is to fill in the data on the above in this way it may be possible to understand how much platelet function deficiency if any is responsible for the bleeding of the patients with congenital F XIII deficiency

Material and Methods

Venous blood was obtained from four patients, aged 4–9 years, with congenital FSF deficiency two of whom have been previously presented [17] Plasma F XIII

Table I. Platelet aggregation in patients with congenital F XIII deficiency by four aggregating agents

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In this study we tried to demonstrate another property of platelet F XIII related to platelet function. Epinephrine- ADP collagen- and ristocetin induced platelet aggregation of these patients with hereditary F XIII deficiency was normal as was the bleeding time (table I). Therefore it is concluded that platelet F XIII does not have any effect on platelet aggregation when these particular aggregants are used.

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Association française des Hémophiles

Le Prix international de l'Association française des Hémophiles sera décerné pour la deuxième fois au cours du 12e Congrès Mondial de l'Hémophilie à New York du 26 juin au 1er juillet 1977 Il est destiné à récompenser les travaux d'un chercheur ou d'une équipe de chercheurs permettant de faire progresser les con-

[illegible] concernant la physiopathologie et la thérapeutique de l'hémophilie ou d'en prévenir le développement.

Les candidatures devront être adressées au secrétariat du Jury: Association française des Hémophiles, Centre national de transfusion sanguine, 6, rue Alexandre-Cabanel, F 75015 Paris (France), au moins 4 mois avant la date prévue pour la désignation du lauréat. Les travaux publiés ou non publiés devront être présentés en langue française ou anglaise, en 10 exemplaires. Ils seront accompagnés d'une note biographique de l'auteur principal et d'un résumé de ses travaux antérieurs en langue anglaise quelle que soit la langue d'origine.

Congress on Immunotherapy of Malignant Diseases

November 9–10, 1977 Vienna, Austria

International Symposium on Cell Separation and Cryobiology

November 10–1., 1977 Vienna, Austria

For more details contact. Dr H. Rainer, c/o Wiener Medizinische Akademie, Alser Strasse 4 *A-1090 Wien* Austria.

International Society of Haematology

The 4th meeting of the European and African Division of the International Society of Haematology will be held in Istanbul on September 5–9 1977 President: Prof. O N Ulutin

The scientific program will include seminars, symposia, round table discussions, workshops, and free communication session. The main topics will be hematopoiesis, hemoglobinopathies, thalassemic syndromes, erythropoietin, iron deficiency anemia and geophagia, lymphocytes, leukemias, malignant lymphomas, platelet structure and chemistry platelet diseases, coagulopathies, fibrinolysis, DIC, thrombosis, antiaggregating drugs, and immunohematology

Submission deadline for papers: April 1st, 1977

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Acta haemat. 56: 321–327 (1976)

Proliferation of Leukemic Blood Cells in Short Term Cultures. Relevance to the Differential Diagnosis of Myeloblastic and Lymphoblastic Leukemia

J. A. J. M. BAKKEREN, G. A. M. DE VAAN and H. F. P. H

Department of Pediatrics (Head: Prof. E. D. A. M. SCH
Division of Hematology (Head: Prof. C. HAANEN), Departm
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Materials and Methods

Patients. 30 patients with ALL (7 children and 3 adults) and 30 patients with AML (10 children and 20 adults) were examined before treatment. 9 patients with ALL (all children) and 8 patients with AML (2 children and 6 adults) were studied with the combination of ³H-thymidine incorporation and pulse cytophotometry. In all patients included in this study the percentage of blast cells in the blood was 50% or more. The diagnosis was confirmed at the WHO Reference Center for Leukemias and Lymphomas at the Institut de Cancérologie et d'Immunogénétique in Villejuif (France) or at the Reference Center of the Dutch Working Group on Leukemia in Children (NWLK) in The Hague (The Netherlands).

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Samples were incubated in quadrifold at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24, 48 and 72 h, ³H-thymidine incorporation was determined, in triplicate, as described previously [1]. 4 h before harvesting, 1 µCi ³H-thymidine was added to the cultures. The cells were collected on the Millipore filter and the incorporation of ³H-thymidine was measured by means of liquid scintillation counting. Results were expressed as cpm per 5×10^6 cells. Cell viability in the cultures was assessed by the trypan blue exclusion method. Cell typing was performed by the differentiation of 200 cells on slides stained with May-Grünwald-Giemsa.

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Table I ^{3}H thymidine incorporation in leukemic cells from patients with ALL and AML, before treatment, after culturing for 72 h

ALL patient No.	total WBC/mm³	cpm/5 × 10⁴ cells	AML patient No.	total WBC/mm³	cpm/5 × 10⁴ cells
1	700	400	1	1,300	1 070
2	5,300	8 190	2	[illegible],150	[illegible],520
3	8,300	740	3	4 500	39,130
4	13 100	640	4	4 700	[illegible],460
5	14 600	[illegible]80	5	8,200	71,300
6	16,400	3,560	6	8,500	4,360
7	[illegible]0,600	630	7	8 700	12,320
8	20 800	780	8	8,700	6,830
9	[illegible],000	[illegible]30	9	8 800	980
10	[illegible]3,500	1 010	10	10,600	38,270
11	4 000	130	11	1[illegible],000	7 700
12	[illegible]5 400	100	12	14 000	11 900
13	26,000	3 430	13	20,700	13,120
14	6,700	560	14	21 800	104,000
15	[illegible]8,500	7 900	15	36,000	2,700
16	35 000	460	16	41 000	3 040
17	35 000	[illegible]70	17	49 000	34 680
18	45,300	1 000	18	53 400	[illegible],030
19	59 000	[illegible],900	19	56,400	8,690
20	60 200	120	20	64 000	23,600
21	66,000	250	21	65 000	6,650
2[illegible]	76,400	1[illegible]0	[illegible]2	65 000	27 600
23	95 400	290	[illegible]3	86,600	6,960
[illegible]4	104 000	270	[illegible]4	128,000	60,000
25	104 000	[illegible]10	25	130,000	78,000
[illegible]6	127 000	150	26	160 000	6,200
[illegible]7	165,000	2,400	27	[illegible]40,000	72,300
28	[illegible]24 000	[illegible],430	[illegible]8	250,000	[illegible],560
29	[illegible]70,000	[illegible],110	29	270,000	63 690
30	300,000	360	30	300,000	4 470
Mean (± SE)		1 400 ± 380			4 640 ± 5 140

$p < 0.0001$ (Wilcoxon's two-sample test).

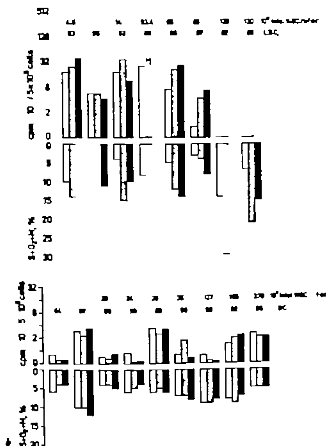


Fig. 1 H-thymidine incorporation (cpm 10*5 10* cells) and cell cycle phase distribution (S+G +M phases, /a) in leukemic cells from untreated patients with AML (a) and ALL (b), after culturing for 24 (white columns), 48 (hatched columns) and 72 h (black columns). LBC = leukemic blood cells

ALL. This could explain her rather low alues for H-thymidine incorporation and (S+G₂+M)-phase percentages, as compared with the other genuine cases of AML. In conclusion, a good agreement was established between the pulse cytophotometric and H-thymidine incorporation data.

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1	700	400	1	1,300	1 070
	5,300	8 190	2	2,150	[illegible]520
3	8,300	740	3	4,500	39,130
4	13 100	640	4	4,700	[illegible]460
5	14 600	[illegible]80	5	8,200	71,300
6	16,400	3,560	6	8,500	4,360
7	[illegible]0,600	630	7	8 700	1[illegible]320
8	20,800	780	8	8,700	6,830
9	[illegible]2,000	[illegible]30	9	8,800	980
10	[illegible]3,500	1 010	10	10,600	38,270
11	[illegible]4,000	130	11	1[illegible],000	7 700
12	[illegible]5 400	100	12	14 000	11 900
13	26,000	3 430	13	20,700	13,120
14	[illegible]6,700	560	14	21 800	104,000
15	[illegible]8,500	7 900	15	36,000	2,700
16	35 000	460	16	41 000	3 040
17	35,000	[illegible]70	17	49 000	34 690
18	45 300	1 000	18	53 400	[illegible]030
19	59 000	2,900	19	56,400	8,690
20	60,200	120	20	64 000	[illegible]3 600
21	66,000	250	21	65 000	6,650
[illegible]	76,400	1[illegible]0	[illegible]	65 000	[illegible]7 600
[illegible]3	95 400	290	[illegible]3	86,600	6,960
[illegible]4	104,000	270	[illegible]4	128 000	60,000
25	104 000	310	[illegible]5	130 000	78,000
26	127 000	150	[illegible]6	160,000	6,200
27	165 000	2,400	27	240,000	72,300
28	224 000	2,430	[illegible]8	250,000	[illegible]560
29	270 000	2,110	[illegible]9	270,000	63,690
30	300,000	360	30	300,000	4 470
Mean (± SE)		1 400 ± 380			24 640 ± 5,140

$p < 0.0001$ (Wilcoxon's two-sample test).

for AML we prefer the *in vitro* ^{3}H thymidine incorporation to the pulse cytophotometric method, because with the first method more clear-cut differences in numerical values are found than for the cell percentages in the pulse cytophotometric method

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serum sources in the culture was examined. From the results in 4 experiments, no consistent differences in the parameters of DNA synthesis were found, whether fetal calf serum, a pool of human AB serum, or autologous serum was used.

Discussion

The incorporation of ^{3}H-thymidine into DNA of a cell population *in vitro* during a 4-hour incubation period can be considered as a measure for its overall DNA synthesis rate. By using the pulse cytophotometric technique the proportion of cells is determined which at any given moment is in the DNA synthesis phase (S phase) of the cell cycle. From the latter assay however no firm conclusions can be drawn about the rate of DNA synthesis of the cell population. By combining both methods, information can be obtained about the proportion of cells that actively synthesizes DNA on the one hand and the rate of this DNA synthesis, on the other hand. The experiments described above indicate that under these culture conditions a larger proportion of the leukemic blood cells from patients with AML is in the DNA synthesis phase as compared with the leukemic blood cells from patients with ALL.

It appeared that the patients peripheral blood leukemic cells, after being brought into culture medium, possessed a much stronger proliferative capacity than was seen in their physiological environment, i.e. the peripheral blood. One can speculate about explanations for this discrepancy. For instance it is conceivable that the high cell concentrations *in vivo* exert a self regulating feed back inhibition on cell proliferation [6, 7]. When these leukemic cells are brought in an *in vitro* system with much lower cell concentrations, this diluting effect would derepress the feedback mechanism and could produce possibly again further cell proliferation. This concept would implicate a characteristic difference between AML and ALL cells. With the latter also in cases with very high WBC counts, proliferation is not encouraged by the same *in vitro* diluting effect. It is quite remarkable that very low rates of ^{3}H thymidine incorporation were found in ALL patients with very high WBC counts, usually associated with massive hepatosplenomegaly. In AML patients this rate was very much higher.

With our data we confirm in a larger series of both AML and ALL patients our earlier report [8] and the suggestion made by BALKWILL *et al* [2] that this type of culture is a real aid to diagnosis. As a diagnostic aid

for AML we prefer the *in vitro* H-thymidine incorporation to the pulse cytophotometric method, because with the first method more clear-cut differences in numerical values are found than for the cell percentages in the pulse cytophotometric method.

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Red Cell Membrane Proteins Abnormality in Paroxysmal Nocturnal Hemoglobinuria and in *in vitro* Induced PNH Like Erythrocytes

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Key Words. Electrophoresis · Erythrocyte · Membrane proteins · Paroxysmal nocturnal hemoglobinuria · PNH-like red cells

Abstract. The abnormality of the red cell membrane proteins, in paroxysmal nocturnal hemoglobinuria (PNH) red cells and in PNH-like ones induced *in vitro* by D-penicillamine and N-acetyl *l*-cysteine, is described. The electrophoresis was effected by a vertical urea starch-gel system. In PNH there is a loss of 5 protein bands and a diminution of one of the remaining bands, and in PNH-like red cells there is a loss of 2 or 3 protein bands.

The red cell membrane in paroxysmal nocturnal hemoglobinuria (PNH) is seriously affected; it is not known, however, if only the function of the membrane or also some of its structural components are altered [2, 9]. The present study describes abnormalities of the electrophoretic pattern of PNH red cells and of PNH-like erythrocytes induced by D-penicillamine (DP) and N-acetyl *l*-cysteine (NALC).

Materials and Methods

PNH red cells were collected from two patients, before any blood transfusion, into ACD, washed 3 times in 0.9% NaCl and used on the same day.

PNH-like red cells were produced *in vitro* by transformation of normal red cells by the method of De Sandre *et al.* [5]. Red cells were collected from normal donors into ACD, washed 3 times in 0.9% NaCl and used on the same day. A 10% water solution of D-penicillamine (Sigma Chemical Co.) and N-acetyl-*l*-cysteine (BDH

Biochemicals Ltd.) was obtained, just before its use, and its pH was adjusted to 8.0 with 1 N NaOH solution. The experimental system consisted of 4 ml of normal packed red cells plus 1 ml of the chemical compound solution. It was mixed in an Erlenmeyer flask under continuous and gentle agitation. The concentration and the chemical characteristics of DP and NALC are given in table I. The chemical compound/red cell mixtures were incubated at 37 C for 30 min for DP and 15 min for NALC. After the incubation, the red cells were washed in 0.9% NaCl. They were sedimented after each wash by centrifugation at 1,500 g for 5 min. After the first wash the red cells were aggregated at the bottom of the tube, and it was necessary to make a new suspension into 0.9% NaCl. The washing of the red cells was continued until the supernatant was free of Hb which was then carefully decanted.

Preparation of membrane proteins. Both PNH red cells and PNH-like red cells were washed 3 times in isotonic phosphate buffer (310 mosm, pH 7.4, 0.1 mM EDTA). All subsequent procedures were carried out at 4 C. The cells were hemolysed and washed in hypotonic phosphate buffer (20 mosm, pH 7.4, 0.1 mM EDTA) until the ghosts became white. The membranes were sedimented after each wash by centrifugation at 30,000 g for 20 min. The hemoglobin-free ghosts were finally washed once with distilled water, resuspended in water and allowed to stand over night [21]. Solubilization of the protein was effected by the method of MADDY [15]. An equal volume of N-butanol (0 C) was added to the membrane suspension, mixed well, allowed to stand for 30 min at 0 C and then centrifuged at 30,000 g for 20 min. The aqueous phase, containing the proteins, was removed with a cold syringe and dialyzed for 12 h against distilled water. It was then lyophilized and stored under vacuum.

Electrophoretic procedures. 20 mg of the lyophilized protein powder were dissolved in 1 ml of a solution of 6 M urea, 3% acetic acid and 0.16 M 2-mercaptoethanol. On standing overnight at 4 C, a clear solution was obtained [1]. The electrophoresis was carried out at an urea starch-gel of pH 3.2 [20]. The gel for this low pH electrophoresis was prepared as follows: 63 g of hydrolysed starch (BDH Biochemicals Ltd.) was powdered together with 180 g of urea (Riedel-DeHaen AG) with pestle and mortar. The gel buffer consisting of 300 ml of sodium lactate (Merck) buffer (pH 3.2, cation concentration 0.05 M) containing 2.4 ml 2-mercaptoethanol, was added to the powder and mixed well before heating to 70 C for 7 min in an Erlenmeyer flask. After degassing, the gel was poured and allowed to set overnight. The protein solution was subjected to electrophoresis for 6 h at an 60mA constant current in a vertical system using the same sodium lactate buffer pH 3.2 of the same cation concentration in the electrode chambers. The gel was stained with amido black 10B and discoloured with 3% acetic acid solution [18].

Results

The electrophoretic pattern of normal red cell membrane proteins consists of 8 bands [6]: the slow moving group of 3 bands (group c), a not always well-defined band, 2 bands in the middle of the electrophoretic

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Table 1 Concentration chemical characteristics and solvent of D-penicillamine and N-acetyl-*l*-cysteine

Chemical compound	Concentration g/100 ml	Formula	Molecular weight g	Time of incubation min	Solvent
D-penicillamine	10	$C_5H_{11}NO_2S$	149..	30	water
N-acetyl-*l*-cysteine	10	$C_5H_9NO_3S$	163.2	15	water

pattern (bands b and a) another not always well-defined band and finally the fast moving band of hemoglobin (Hb) (N in fig. 1 2). The electrophoretic pattern of the PNH red cell membrane proteins consists only of bands a and the band of Hb and of a diminished b band (PNH 1 and PNH 2 in fig. 1) The electrophoretic pattern of the PNH like red cell membrane proteins, after treatment of normal red cells with DP consists of the group c bands, the b a and Hb bands (fig. 2, DP) and after treatment with NALC, of 2 out of 3 group c bands and of the b, a and Hb bands (fig 2, NALC)

Discussion

Many abnormalities of the red cell membrane proteins have been shown in various diseases [7 8, 12] and in *in vitro* experiments [13] The red cell membrane protein electrophoresis [14 16 17] is a new method for the evaluation of the erythrocyte membrane alterations. The present study shows that in PNH the electrophoretic pattern is rather abnormal because of the loss and the modification of the protein bands. This finding is in agreement with the findings of other workers [11] using rather different methods.

Normal red cells transformed *in vitro* to PNH like ones by DP and NALC gave positive acid serum, sucrose and thrombin tests, that is they were lysed under the same conditions as PNH erythrocytes [3 4 10 19] Their membrane protein electrophoretic pattern however is quite different from that of the PNH red cells, showing slighter modifications. The action of DP and NALC on normal red cells alters principally their membrane functions and in a lesser degree their proteins. The yet unknown

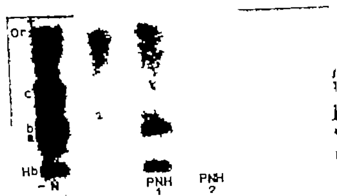


Fig. 1 Electrophoretic patterns of PNH red cell membran... of patients (PNH 1, PNH 2) and of normal red cells (N). Or = origin ... bands c; b = band b; ... = band a, Hb = Hb band.

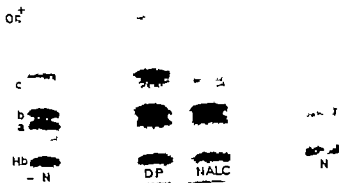


Fig. 2. Electrophoretic patterns of PNH-like red cell membrane proteins, that is normal red cells treated with D-penicillamine (DP) and N-acetyl-l-cysteine (NALC), and of normal red cells (N). Or = origin ... = group of bands c; b = band b; ... = band a, Hb = Hb band.

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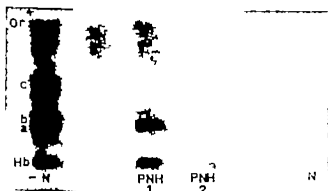


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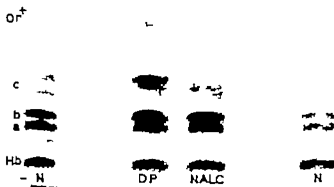


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Table I

Dithionite test	Hemoglobin electrophoresis A	AS	S	AC	total
+	28	39	4		71
	309	9		2	320
Total	337	48	4	2	391

Subjects and Methods

The study group consisted of adult Saudi males arying in age from 16 to 30 years bo ere undergoing rigorous pre-employment health assessment through the medical department of the Arabian American Oil Company in Dhahran, Saudi Arabia. A venous blood sample anticoagulated with EDTA as obtained from each subject, and screening for hemoglobin S and glucose-6-phosphate dehydrogenase (G6PD) was performed on each sample within 24 h of collection. Results of G6PD screening and electrophoresis are subjects of separat eport. 50- l aliquots from each sample were spotted on Whatman No. 1 filter paper strips hich ere placed in sealed plastic bags containing silica gel and stored at 4 C until shipment to the United States for electrophoretic analysis. A modification of the dithionite method [4] for identifying hemoglobin S was used as the screening test, and upon receipt in the USA blood samples on filter paper strips ere stored at 20 C until elution and electrophoretic analysis for hemoglobins and G6PD in Tris-borate EDTA buffer at pH 9.1 according to the technique of SPARKES *et al.* [13].

Results

Blood samples were obtained from 391 subjects over a 3-week period. Although hemoglobin electrophoretic patterns obtained from the filter paper eluates were judged to be sharp and discriminating for all subjects tested, there were notable discrepancies between the results of dithionite screening and hemoglobin electrophoresis (table 1). A total of 52 subjects were foud to have an AS or S pattern on electrophoresis, and of this number 43 had corresponding positive dithionite tests. 28 additional subjects had positive dithionite tests in the absence of electrophoretic confirmation. Under the conditions of this study w th hemoglobin electrophoresis as the reference method, the sensitivity of the dithionite tests was 83 and

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Screening for Abnormal Hemoglobins in the Middle East New Data on Hemoglobin S and the Presence of Hemoglobin C in Saudi Arabia[1]

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Key Words Dithionite test Hb C Hb S Hemoglobinopathies Sickle cell disease

Abstract 48 of 391 apparently healthy adult Saudi males had an AS hemoglobin pattern on electrophoresis, four subjects had an S pattern, and two subjects AC. A dithionite screening test lacked sensitivity and specificity in detecting hemoglobin S under the conditions of this study A sickle cell trait frequency of 0.123 evidenced by hemoglobin electrophoresis correlates closely with previous studies of hemoglobinopathies in Saudi Arabs. The presence of four subjects with sickle cell disease in the study group provides further evidence of the benign nature of sickle cell disease in Saudi Arabia.

The high frequency of the sickle cell trait and the occurrence of comparatively mild sickle cell disease in Saudi Arab oasis populations have been subjects of a number of recent publications [3–5 11] The investigation herein reported was designed to identify abnormal hemoglobins not disclosed by previous surveys, to estimate the prevalence of benign homozygous sickle cell disease in Saudi Arabia to evaluate a newer screening technique for identifying hemoglobin S to determine the utility of storing blood samples on filter paper in the field for later hemoglobin electrophoresis and to compare the current prevalence of the sickle cell trait with that observed in the previous surveys of the Saudi population.

[1] This study was supported i part by a grant from the Arabian American Oil Company Dhahran, Saudi Arabia.

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the specificity 92%. Four subjects with apparent homozygous sickle cell disease were identified by electrophoresis, and all four had positive dithionite tests. There were two subjects demonstrating an AC electrophoretic pattern.

Comment

The frequency of the sickle cell trait in Saudi Arabs of 0 123 found in this study compares very closely with that observed in two earlier studies which provided trait frequencies of 0 115 and 0 125 respectively [9 6] The excessive number of apparent false-positive tests with the dithionite screening method is difficult to explain but may either be due to technical problems in the preparation of reagents and performance of the test under field conditions, or alternatively to the occurrence of nonspecific precipitation of non hemoglobin proteins under conditions of high salt (phosphate buffer) concentration [1] The limited sensitivity of the test is not likely to reflect a significant frequency of hemoglobin D (which migrates as hemoglobin S at a pH of 9 1) in this population sample not a single example of hemoglobin D was encountered in the large survey conducted by LEHMANN *et al* [9] The dithionite test may not detect hemoglobin S in the low percentage which frequently characterizes expression of the sickle cell trait in Saudi Arabs [6]

Of interest is the occurrence of homozygous sickle cell disease (SS) in 4/391 apparently healthy young males. With a sickle cell trait frequency of 0 123 the expected number of abnormal homozygotes would be 391 (0 061) or 1–2 individuals The significance of this finding awaits the results of a more extended survey now in progress. The presence of hemoglobin C in the Saudi population is not unexptected in view of the prevalence of hemoglobin S historical evidence of African gene flow to the Middle East [7] and the high frequency of African blood group markers in the Saudi population [10] Hemoglobin C is likely to be found in any Middle East locality where the genetic composition of the inhabitants has been influenced by the East African slave trade [12]

Consonant with other published observations [2, 13] storage of blood samples on filter paper for electrophoretic screening appears to be a practical and reliable technique for field surveys of hemoglobinopathies. The dithionite test without further modification is not currently dependable for detecting hemoglobin S in surveys of the Saudi Arab population

seen. In a recent report of 16 patients transplanted, 14 experienced remissions and all showed recovery of marrow function following high doses of chemotherapy and supralethal doses of irradiation[4]. Prior to this study an additional 5 patients were reported all of whom had marrow reconstitutions [5].

Recently we used the same approach in a patient with acute myelogenous leukemia refractory to chemo- and immunotherapy. Engraftment was not achieved although he survived 44 days and received two marrow infusions.

Case Report

The patient was a 53-year-old man who was found to ha acute erythroleukemia in August 1974. Treatment was started ith cytosine arabinoside (Ara-C) and daunorubicine (protocol No. 7421/III/Induction, ALGB). H developed severe pancytopenia and was treated with broad-spectrum antibiotics. Also, he was supported ith red cell, granulocyte [6] and platelet transfusions. His identical t in brother served as donor of 4 granulocyte and 5 platelet transfusions. The patient achieved complete remission and as placed on maintenance program including Ara-C 6-thioguanine (6-TG), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea (CCNU), cyclophosphamide (CY), and immunotherapy in the form of ural oncolysates given intravenously and subcutaneously (the ural oncolysates ere obtained from PD Dr CH. SAUTER, Department of Oncology and Hematology Kantonsspital, Zurich) (modified protocol No. 7421/A/Maintenance, ALGB). T eeks after the second dose of oncolysate elatively low titer (1 80) of antibodies directed against the strain of fowl plague irus used i the production of the oncolysate were detected in the serum.

I January 1975 there was clear-cut evidence of relapse. On admission the patient complained of fatigue. Physical examination revealed slightly enlarged spleen. Hematocrit 34 vol%/ ith 10% reticulocytes, platelet count 39,000 mm³ hite blood cell count 2,300 mm³ with 5% granulocytes and 73% blasts. Marrow examination showed almost complete replacement with blast cells of acute myelogenous leukemia. No evidence of fibrosis was found on marrow biopsy. Blood chemistries were normal except for SGPT of 48 U (normal 5–27 U). An EKG showed nonspecific T av changes.

I preparation for engraftment he was placed in 'lif island' and gi en oral nonabsorbable antibiotics and mycostatics, sterile diet and skin cleansing. H as treated ith combination chemotherapy and total body irradiation (TBI). For convenience the day of marrow infusion is day 0. H as given vincristine 2 mg on day 9 CY 45 mg kg on day –8, Ara-C 150 mg q 1 h f om day –8 to day 3, 6-TG 100 mg p.o. q. 12 h from day –8 to day 3 and busulfan 10 mg p.o. qd day 9 to day –4. On day 1 he was given 1,000 rad of TBI from linear accelerator at dose rate of 7.5 rad min

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Failure of Isogeneic Marrow Engraftment in a Patient with Acute Leukemia[1]

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Key Words. Acute myelogenous leukemia · Bone marrow transplantation · Failure of bone marrow engraftment · Interferon and inhibition of hemopoesis · Isogeneic marrow transplantation · Leukemia

Abstract. Marrow transplantation was carried out in a patient with acute myelogenous leukemia using a healthy identical twin as donor. The patient was prepared for grafting with combination chemotherapy, high dose cyclophosphamide, and 1,000 rad total body irradiation. Repopulation of marrow did not occur despite a second marrow infusion 21 days following the first attempt. The patient died 44 days after the initial marrow infusion. To our knowledge this is the first patient transplanted from an identical twin who did not achieve engraftment after high dose chemotherapy and supralethal irradiation. The survival time was well in excess of that required for evaluation of engraftment. The possible mechanisms for graft failure are discussed. The use of prophylactic interferon against viral infections is considered as the most likely cause of failure of engraftment.

Rodent studies have demonstrated that syngeneic marrow will invariably repopulate lethally irradiated animals if they survive long enough for stem cell differentiation [1]. In large animal studies it was shown that fresh or frozen autologous marrow will rescue animals from lethal irradiation injury [2, 3]. This has prompted studies to treat patients with leukemia who have identical twins with total body irradiation or a combination of chemotherapy and total body irradiation.

In man, recent studies have demonstrated that this approach invariably results in marrow repopulation and long-term remissions are often

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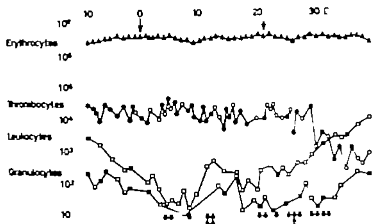


Fig. 1 Hematological events after transplantation ● = Platelet transfusions, ▲ = red cell transfusions; ■ = granulocyte transfusions, ↓ = transplantation.

of normal hematopoetic precursor cells with occasional mononuclear cells with nucleoli and with occasional reticulum cells. In the peripheral blood there were consistently more than 100 leucocytes with up to 90% blasts. Clinical problems during the first 21 days consisted of severe progressive mucositis and severe mental depression. The patient required frequent platelet transfusions and was treated with broad-spectrum antibiotics for fever without documented infection. A second marrow infusion of 11×10^9 nucleated cells (= 1.6×10^8 cells/kg) was performed on day 21. His condition steadily worsened with severe mucositis progressing to swelling of the mouth, pharynx, culminating in respiratory arrest on day 22. He was intubated first by the endotracheal route and subsequently by tracheostomy in order to alleviate upper airway obstruction due to edema and/or inflammation. He developed bilateral progressive pneumonitis and expired 44 days after the initial marrow infusion.

At autopsy we found acute myelogenous leukemia infiltrating all the examined bones, the lungs, the spleen, the para-aortal, paratracheal and bifurcational lymph nodes. There was mild radiation fibrosis of the vertebral bodies. The lungs showed generalized interstitial and intra-alveolar pneumonitis. Pneumocystis carinii was demonstrated histologically. There was excentric cardiac hypertrophy and dilatation with subepicardial and subendocardial hemorrhages. Hemorrhages were found in the thyroid, kidneys, trachea and pleural cavity. The brain was edematous. The esophagus showed candidiasis.

During the posttransplantation period the patient had received a variety of drugs including: cephalotin, gentamycin, carbenicillin, lincomycin, amphotericin-B, 5-fluorocytosine, trimipramine, interferon (supplied by Dr G. Emödi, Kinderspital, Basel), pethidine, novaminsulfon, morphine, diazepam, pentazocine, bromhexine and glucocorticosteroids.

Table I. Probabilities of the blood groups observed, when the twins are either monozygous or dizygous (for this calculation the blood group frequencies in the Netherlands were used)

Blood group system	Monozygous (X)	Dizygous (Y)	X/Y	Products of quotients X/Y
ABO	0.3509	0.2273	1.5438	1.5438
Rhesus	0.3444	0.1872	1.8397	[illegible]8401
MNS	0.[illegible]40	0.1066	2.1013	5.9680
P	0.2116	0.11[illegible]8	1.8759	11.1954
K	0.9149	0.8755	1.0450	11.699
Fy	0.3600	0.[illegible]304	1.5625	18.2800
Lu	0.9312	0.8989	1.0359	18.9368
Lewis (secretor)	0.[illegible]304	0.1[illegible]62	1.8[illegible]57	34.5714
Jk	0.[illegible]601	0.1483	1.7539	60.6340
Gm	0.4[illegible]57	0.2871	1.4828	89.9056
Inv (Km)	0.1719	0.0990	1.7364	156.1058
HL A	0.000351	0.000094	3.7340	58[illegible].9169
Sex	0.50	0.25	[illegible].0000	1 165.8339
Overall relative frequency of monozygous and dizygous	0.[illegible]5	0.75	0.3333	338.61

Drugs and irradiation were reasonably well tolerated except for clinical signs of right heart failure following the CY and the occurrence of nonspecific T wave inversions on the EKG. Therefore he was not given a second dose. On 1/17/75 he was given 10×10^9 nucleated marrow cells ($\approx 1.4 \times 10^8$ cells/kg) from his identical twin obtained by techniques previously described [5].

Identity of the twin brother was established within reasonable limits by family history, HL A identity, identity for 18 Ig-allotypes and for 12 red cell antigens. The probability of the twins to be monozygous was 99.74% (table I). There was a past history of lumbar disc prolapse. In the year prior to transplantation the donor did not take any medication. Physical examination was normal, however, it had been noted previously that he had borderline to definitely low peripheral white blood cell and platelet counts. His hematocrit was between 35 and 39 vol% with 0.7% reticulocytes, white blood cell count between 2,200 and 4,100 cells/mm³ with 55–80% granulocytes, platelet count between 83,000 and 175,000/mm³. Repeated marrow aspirates from the donor did not show any conclusive abnormalities. Progenitor cells assayed *in vitro* in a methylcellulose system were borderline low when stimulated with erythropoetin and colony stimulating activity in a first set of studies but were found to be normal in a second one.

The hematological events after transplantation are shown in figure 1. There was no evidence of engraftment. Marrow aspirations on day 6 and 18 revealed absence

of 10^9 allogeneic leukemic cells infected with a strain of fowl plague virus [11]. This virus is considered to be apathogenic for man [1]. Failures of the hemopoetic inductive microenvironment has been considered as a cause of failure of engraftment and of marrow aplasia. This has been subject to a recent review [13]. Most patients who had successful isogeneic grafts for hemopoetic malignancies had similar combinations of TBI and chemotherapy as ours, and this did not prevent the induction and the support of hemopoesis. Although it is known that amphotericin B and 5-fluorocytosine can suppress marrow function [14] the cruc al point probably is that our patient was given prophylactic interferon against iral infec tion. We have seen that the allogeneic grafts that are gi en prophylactic interferon need platelet and granulocyte support much longer than usual [15] and also we have documented a dose-dependent inh biting effect of interferon on colony growth *in vitro* [16]. This cytotoxic ffect seems to be species-specific since it was not found in similar experiments with rabbit marrow [16]. The interferon preparation available fo clinical use is only partly purified. So far it is not known whether the inhibition is due to interferon itself or to a cytotoxic by product of the interferon-producing cells. Further experiments with partly purified control substances are under way [17].

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Discussion

It is difficult to be sure what the cause of failure of engraftment of this isogeneic marrow transplant is, which to our knowledge is the first one in a patient with AML prepared with chemotherapy and total body irradiation. He presented with acute erythroleukemia which under chemoimmunotherapy maintenance went into AML. Both types of leukemia are considered to be cellular diseases and are not believed to be due to disturbances of the marrow matrix or humoral factors which might interfere with engraftment. Persistent leukemia was the most likely cause of the recurrence but it does not explain the complete lack of a take. A malignant transformation of the transplanted cells had only been reported in two allogeneic grafts for ALL where TBI alone was used for conditioning [7, 8]. Both of these patients had at least transiently experienced engraftment with normal stem cells documented by rising blood counts of all cell lines. It was therefore postulated that possibly an agent present in the host was responsible for the leukemic transformation of the engrafted normal marrow [8].

Persistent leukemia may have suppressed marrow function to some extent, but the marrow examinations performed on day 6 and 18 showed a virtually aplastic marrow with only scattered mononuclear cells, which makes it very unlikely that there was a space problem for proliferation of the graft. The survival time of 44 days certainly seems to be sufficient to evaluate isogeneic engraftment. It is unlikely that the cell number of the graft was too small for a take. The quantity of marrow resulting in successful allogeneic engraftment ranged between 1.1 and 10.9×10⁸ nucleated marrow cells/kg of recipient body weight [9]. It has been shown that for isogeneic grafts the cell number needed for engraftment is smaller [1].

We do not have a definite answer for the borderline low peripheral blood counts of the donor. The fact that he had normal CFU-C and later controls showed normal blood counts and normal marrow argues in favor that he was hematologically normal and that we had given sufficient marrow for successful engraftment. Viral infections can cause transient cytopenias. In our renal transplantation unit they have been considered to be the cause of a series of acute kidney rejections [10]. In failures of marrow grafts to take they have not as yet been considered seriously.

It is unlikely that the active immunotherapy maintenance the patient was placed on, had an influence on the graft take. He had a total of 3 ml of viral oncolysate. Each milliliter contained the approximate equivalent

of 10^9 allogeneic leukemic cells infected with a strain of fowl plague virus [11]. This virus is considered to be apathogenic for man [12]. Failures of the hemopoetic inductive microenvironment has been considered as a cause of failure of engraftment and of marrow aplasia. This has been subject to a recent review [13]. Most patients who had successful isogeneic grafts for hemopoetic malignancies had similar combinations of TBI and chemotherapy as ours, and this did not prevent the induction and the support of hemopoesis. Although it is known that amphotericin-B and 5-fluorocytosine can suppress marrow function [14] the crucial point probably is that our patient was given prophylactic interferon against viral infection. We have seen that the allogeneic grafts that are given prophylactic interferon need platelet and granulocyte support much longer than usual [15] and also we have documented a dose-dependent inhibiting effect of interferon on colony growth *in vitro* [16]. This cytotoxic effect seems to be species-specific since it was not found in similar experiments with rabbit marrow [16]. The interferon preparation available for clinical use is only partly purified. So far it is not known whether the inhibition is due to interferon itself or to a cytotoxic by-product of the interferon-producing cells. Further experiments with partly purified control substances are under way [17].

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Unusual Clonal Evolution in a Case of Chronic Myelogenous Leukemia

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Key Words. Cell clones in leukemia · Chronic myelogenous leukemia · Karyotype · Leukemia · Marker chromosomes · Ph chromosome · Translocation

Abstract. Several unusual cytogenetic changes have occurred during the evolution of chronic myelogenous leukemia in 32-year-old hite male ith this disease for 8 years. The first appearance of hypodiploid cell line containing dicentric marker occurred 2 years after diagnosis and this line was eliminated by several courses of therapy with hydroxyurea. A second clone, which had partial deletion of the long arm of one of the number 8 chromosomes (8q–) was noted year later but this line has been refractory to intensive combination chemotherapy.

Karyotypic changes in the Ph¹-positive cell population in patients with chronic myelogenous leukemia (CML) during progression to blastic transformation have been well recognized [3, 5, 6, 12–14, 16, 18, 19, 23, 24]. In this paper we wish to describe a patient who developed several unusual cytogenetic changes during the evolution of his illness, and to correlate these changes with his clinical course and treatment.

Case Report

P. H. is 32-year-old white male who had chronic myelogenous leukemia diagnosed in 1968 and eferred to the National Cancer Institute in November of 1969. Physical examination at that time was unremarkable except for palpable spleen tip. Laboratory data are included in table I: hemoglobin 13.2 g%, white blood count 51,400/mm³ with no myeloblasts in the peripheral blood, and platelet count 202,000/mm³. A bone marrow examination revealed hypercellular marrow with

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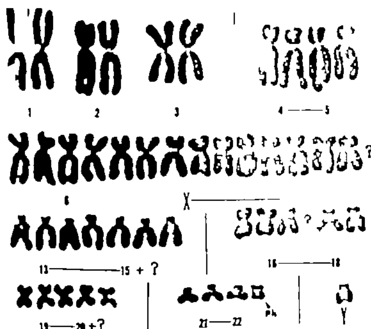


Fig. 2. A karyotype of pseudodiploid cell (Giemsa stain of bone marrow preparation of September 11, 1972) showing missing C group and E group chromosomes, extra D group and F group chromosomes, and one Ph chromosome

time, the WBC had risen to 43,500/mm³ and there were 2% myeloblasts. Bone marrow examination revealed 4% myeloblasts. Cytogenetic evaluation showed that the clone of cells having the dicentric marker was found for the last time, and the one containing the 8q- marker had become dominant.

In May 1975, the patient had an abrupt increase in white count and in peripheral myeloblasts to approximately 83%. The hemoglobin dropped to 9.8 g%, and the patient began developing toxic fevers without known source of sepsis. In June, bone marrow biopsy showed diffuse infiltration with myeloblasts and cytogenetic studies were not possible. However, all the cells in the peripheral blood had the 8q- marker chromosome. This blastic transformation phase was treated with several courses of cytosine arabinoside and 6-thioguanine without success. Therapy was then changed to several courses of vincristine and prednisone which has also failed to induce reversion to the chronic phase. A 24-hour intravenous infusion with high dose of methotrexate (600 mg) was then used with definite improvement noted. However this response was very brief and the patient expired April 2, 1976. Autopsy permission was denied.

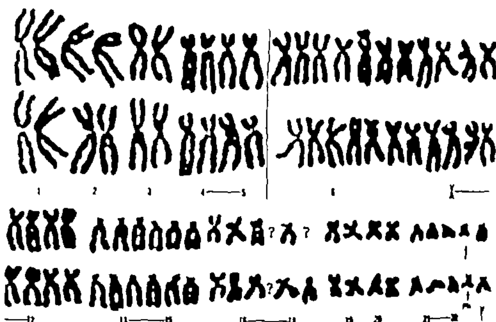


Fig 1 Two karyotypes (Giemsa stain) from a bone marrow preparation (November 1971) showing the dicentric marker and the Ph chromosome.

3% myeloblasts. Leukocyte alkaline phosphatase was 48 (normal). In February 1971, he was treated with busulfan because of progressive leukocytosis to approximately 100,000/mm³ and a fall in the platelet count to 110,000/mm³ and splenomegaly. He underwent an elective splenectomy in April of 1971. Laboratory data of September 30, 1971: serum B12 1 780 pm/ml (normal 170–760 pm/ml), white blood cell count 7 100/mm³, hemoglobin 15.5 g%, and platelet count 124 000/mm³. No myeloblasts were seen in the peripheral blood and bone marrow analysis revealed less than 1% blasts. A clone of hypodiploid cells with a dicentric chromosomal marker was noted for the first time (fig. 1). Busulfan was discontinued on December 10, 1971 at a time when the patient was clinically stable.

The patient's course was unremarkable until August, 1972, when his white blood count again began to increase, reaching 13,800/mm³ with no myeloblasts on the peripheral smear. Cytogenetic analysis revealed two clones of cells with separate markers, and one pseudodiploid cell which had 46 chromosomes and one Ph chromosome, but with one extra chromosome each in groups D and F and a missing chromosome each in groups C and E (fig. 2). The dominant cell was hypodiploid with a dicentric marker while the other line had the 8q− marker.

By June 1973, the patient's WBC had risen to 4[illegible],000/mm³ with 4% myeloblasts and he was started on 1 g/day hydroxyurea which was gradually increased to 4 g/day. After the WBC was lowered to about 10,000/mm³, hydroxyurea was discontinued and the patient remained clinically stable until January 1974. By thi

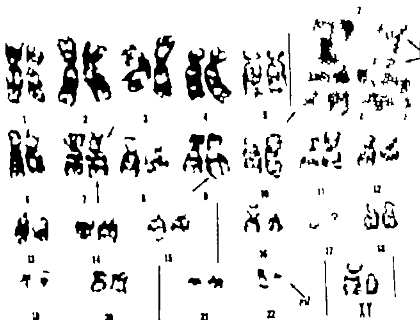


Fig. 3. A karyotype (trypsin G-banding) with 45 chromosomes showing the translocation of the second Ph chromosome to the number 7 chromosome, 9q and the Ph chromosome. The enlarged chromosomes show normal number 7 and the dicentric marker with the arrow indicating the centromeres of the 7 and the Ph.

and one Ph chromosome; but it had one extra chromosome in each of groups D and F and was missing one chromosome each in groups C and E (fig. 2). One cell was seen which had a smaller C group chromosome. The remainder of the cells had 46 chromosomes including one Ph[1] chromosome. No definite clone could be identified in those metaphases with 44 chromosomes and the dicentric marker. In February 1973, G-banding showed that the dicentric marker in the cells with 45 chromosomes resulted from a translocation of a second Ph chromosome to a partially deleted short arm of one of the number 7 chromosomes (7p–) (fig. 3). In addition, there was elongation of the long arm of one of the number 9 chromosomes (9q+) and one Ph chromosome; that is, 45,XY,Ph[1] t(9,22) (q34,q12), dic(7,Ph) (p1 p13). On January 7

Table I Serial cytogenetic and hematological data

Date	Specimen	Chromosome number (100% Ph¹-positive)							Clinical status	Peripheral blood				
		39–43	44	45	46	48	tet	total		BM blasts %	WBC × 10³	blasts %	Hb g%	platelets × 10³
11 24–69	BM				15			15	chronic phase	3	51 4	0	13._	202
09–30–71	BM	1		11[1]	27	1		40	chronic phase	0	7 1	0	15.5	1 4
09–11–72	BM	1,5[1]	13[1]	50[1]	13 1[2]			8_	chronic phase	3	13.8	0	10.6	49
02 _6–73	BM	_[1]	11[1]	48[1]	10[2]			61	accelerated phase	4	35 1	2	14.5	532
01–07–74	BM	1	5	9[1]	3[2]			18	chronic phase	4	43.5	2	1_9	1156
01–07 75	BM		1		8[2]		1	10	chronic phase	1	41 9	6	12.9	430
06–30–75	PB	2	4	4	73[2]			80	blast crisis[3]		97 0	90	9.8	603
08– 6–75	PB				9[2]			9	blast crisis[3]		_6.3	71	10.5	949
	BM	no mitoses							(fibrotic marrow)					

[1] Dicentric marker chromosome.
[2] 8q– marker chromosome.
[3] Diffuse infiltrate of myeloblasts.

Cytogenetic Findings

Serial cytogenetic studies were carried out on 1 2 and 3-day peripheral blood cultures and direct bone marrow preparations without prior *in vitro* culture Only conventional Giemsa stain was available for the studies prior to September 30 1971 subsequently both conventional and trypsin-Giemsa banding stains were done Results of cytogenetic studies and relevant hematologic data are shown in table I In February 1973 G banding showed that the dicentric marker in the cells with 45 chromosomes resulted from a translocation of a second Ph¹ chromosome to a partially deleted short arm of one of the number 7 chromosomes (7p–) (fig. 3) In addition there was elongation of the long arm of one of the number 9 chromosomes (9q+) and one Ph chromosome that is, 45 XY Ph t(9 22) (q34 q12) dic(7 Ph) (p12 p13) On January 7 1974 G banding made it possible to identify (September 30 1971) another cell population in the bone marrow one third of the metaphases had 45 chromosomes including one Ph chromosome and a dicentric marker chromosome (fig 1) By September 11 1972 80% of the cells contained this marker One cell was pseudodiploid with 46 chromosomes

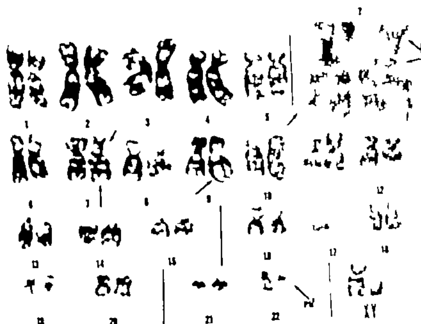


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and one Ph chromosome but it had one extra chromosome in each of groups D and F and was missing one chromosome each in groups C and E (fig. 2). One cell was seen which had a smaller C group chromosome. The remainder of the cells had 46 chromosomes including one Ph chromosome. No definite clone could be identified in those metaphases with 44 chromosomes and the dicentric marker. In February 1973, G-banding showed that the dicentric marker in the cells with 45 chromosomes resulted from a translocation of a second Ph chromosome to a partially deleted short arm of one of the number 7 chromosomes (7p–) (fig. 3). In addition, there was elongation of the long arm of one of the number 9 chromosomes (9q+) and one Ph chromosome that is, 45,XY,Ph t(9,22) (q34,q12), dic(7,Ph) (p12;p13). On January 7



Fig 4 Two karyotypes (trypsin G banding) from a bone marrow of January 1974 showing the 8q− marker 9q+ and the Ph chromosome.

1974 banding patterns made it possible to identify the smaller C group chromosome which had appeared 1 year earlier This chromosome was number 8 with a partial deletion of the long arm (8q−) in addition, these cells had 9q+ and one Ph[1] chromosome (fig 4) that is, 46,XY Ph[1] t(9,22) (q34 q12) 8q23− The unusual dicentric marker was last seen in this bone marrow specimen Since this time the patient's marrow has been hypoplastic and metaphases which could be studied had 46 chromosomes including one Ph[1] chromosome No successful bone marrow preparations for cytogenetic analyses have been possible since January 1975 and all karyotypes after this date were from peripheral blood cultures with or without PHA stimulation. Metaphases from these samples which had 46 chromosomes and one Ph chromosome, had 8q− also

Discussion

In cytogenetic studies from the beginning of this patient s ill ess, all the cells of his bone marrow had 46 chromosomes with a si gle Ph chromosome. At that time, banding techniqu s were not available bu analyses of metaphases from conventional Giemsa stained prepar i showed that the Ph was the only abnormality pr sent n these cel r 2 years, the patient remained stable, then deve ped p nomegal thrombocytopenia. Cytogenetic evaluation of th h m at time (September 30 1971) showed two cell li e h 4 ch i somes with a dicentric marker chromosome and th h mosomes with one Ph chromosome. Shortly after other populations were noted with development of d somal abnormalities, that is, the appearance of se r cells. The cells with 8q– seemed to have some selecti these have become the predominant cell type later in the di

A high incidence of abnormalities in olving chromoso and 8 have been previously reported in a variety of hem c cases. Monosomy 7 was observed in erythroleukemia [20 leukemic phase of myeloblastic leukemia [11] and in patients w th loproliferative syndromes [15 21] while trisomy 7 has occur d chronic myelogenous leukemia [9] In addition, trisomy of chromo n number 8 has been reported in patients with hematological disorders [7 8], and in 6 cases of chronic myelogenous leukemia [22] This sug gests that the extra or missing chromosomes number 7 or 8 may play an important role in regulating the differentiation of granulocytic precur sors.

The development of a cell population with a second Ph chromosome in advanced cases of CML is well recognized [4 10 17] We have re- ported an earlier series of CML cases in which 15 of the 32 patients who orignally had either 46 Ph¹-positive cells or both 46 Ph¹-positive and negative cells developed double Ph chromosomes in the late stages of their disease [25]. Our present case had a clone which also gained a second Ph¹ chromosome but in a rather unusual way the additional Ph chromosome was translocated to chromosome number 7 Other cases have been reported where a dicentric chromosome was formed by two Ph¹ chromosomes [26] but there has been no report of a second Ph chromosome translocating to chromosome number 7 In table I, it can be seen that the hypodiploid cell line (those cells with 45 chromosomes)



Fig 4 Two karyotypes (trypsin G-banding) from a bone marrow of January 1974 showing the 8q− marker 9q+ and the Ph¹ chromosome.

1974 banding patterns made it possible to identify the smaller C group chromosome which had appeared 1 year earlier This chromosome was number 8 with a partial deletion of the long arm (8q−) in addition, these cells had 9q+ and one Ph chromosome (fig. 4) that is, 46,XY Ph¹ t(9,22) (q34 q12) 8q23− The unusual dicentric marker was last seen in this bone marrow specimen Since this time the patient's marrow has been hypoplastic and metaphases which could be studied had 46 chromosomes including one Ph¹ chromosome No successful bone marrow preparations for cytogenetic analyses have been possible since January 1975 and all karyotypes after this date were from peripheral blood cultures with or without PHA stimulation Metaphases from these samples which had 46 chromosomes and one Ph¹ chromosome had 8q− also

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In cytogenetic studies from the beginning of this patient's [illegible], all the cells of his bone marrow had 46 chromosomes with a single Ph chromosome. At that time, banding techniques were not available, but analyses of metaphases from conventional Giemsa stained prepar[illegible] showed that the Ph was the only abnormality present in these cel[illegible] 2 years, the patient remained stable, then developed [illegible] thrombocytopenia. Cytogenetic evaluation of th[illegible] time (September 30 1971) showed two cell li[illegible] somes with a dicentric marker chromosome, and th[illegible] mosomes with one Ph chromosome. Shortly after other populations were noted with development of [illegible] somal abnormalities that is, the appearance of se[illegible] cells. The cells with 8q− seemed to have some selecti[illegible] these have become the predominant cell type later in the di[illegible]

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appeared at around September 30 1971 but had disappeared by April 15 1974 This is similar to the case reported by CANELLOS et al [1] in which a hypodiploid cell line associated with blastic transformation disappeared when remission was induced by therapy with vincristine and prednisone. The transient remission in this patient as well as in the previous one was accompanied by a return to the diploid chromosomal constitution which usually characterizes the chronic phase of the disease. This suggests that the hypodiploid cell line seen in some CML patients in blastic crisis may represent a more favorable form of blastic transformation as these cells are relatively sensitive to chromotherapeutic agents, and therapy may result in obliteration of the cell line with a return to a chronic phase In contrast, the present dominant clone of pseudodiploid cells having 46 chromosomes with the 8q– seems very refractory to treatment. This clone has now replaced all other bone marrow cell populations, and as a result, this patient is currently in continuous blastic phase Serial determinations of cytogenetics in such patients may result in early characterization of new blast cell lines, in terms of sensitivity to various therapeutic regimens and providing important prognostic and therapeutic information allowing more appropriate therapy in the accelerated and blastic transformation phases of CML.

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Primary Fibrinolysis and Spontaneous Rupture of the Spleen in Acute Lymphoblastic Leukemia

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Department of Medicine B and Hematology Clinic, Hasharon Hospital, Petah-Tiqva, and Tel Aviv University Medical School, Tel Aviv

Key Words. Fibrinolysis · Leukemia · Lymphoblastic leukemia · Spleen rupture

Abstract. A 51-year-old male with acute lymphoblastic leukemia whose course was complicated by primary fibrinolysis and spontaneous rupture of the spleen is described. The patient was treated with various drug combinations: vincristine and prednisone, later by cytosine arabinoside and finally by prednisone, methotrexate and 6-mercaptopurine. Four months after the diagnosis he developed epistaxis and petechiae. The coagulation tests were compatible with primary fibrinolysis and he responded to treatment with -aminocaproic acid (EACA). One month and half later he developed again epistaxis, ecchymoses, splenomegaly and an acute abdomen. The coagulation tests revealed primary fibrinolysis. The operation revealed ruptured spleen. After splenectomy the treatment with EACA was continued and, despite an improvement in the coagulation tests, the patient died.

The common complications of acute leukemias are anemia, hemorrhage, usually secondary to thrombocytopenia, infections, urate nephropathy, leukemic meningitis and bone involvement. Overt fibrinolysis in acute lymphoblastic leukemia is an uncommon complication. We report a patient with acute lymphoblastic leukemia whose course was complicated by primary fibrinolysis and spontaneous rupture of the spleen.

Case Report

E. M., 51-year-old male, was admitted to the department on December 10, 1974 because of marked weakness and prostration of several weeks' duration. The patient complained of left hypochondrial fullness and left shoulder pain. His past

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Table I. Results of the coagulation studies

	Normal values	Patient's values				
		10.12.74	8.4.75	11.4.75	20.5.75	.1 5.75
Platelets/μl	150 000–400,000	45,000	40,000	35,000	27 000	48,000
Fibrinogen, mg %	200–400	250	110	160	80	260
Prothrombin time, %	100	80	30	60	22	20
Partial thromboplastin time, sec	30–35	31	48	35	75	65
Thrombin time, sec	9–13	10	30	8	70	13
Euglobin lysis time, min	>120	140	30	130	20	30
Whole blood lysis time, h	>24	>24	1.5	>24	0.5	2
Fibrinogen degradation products, μg/ml	<10	10	>40	>10	>10	>10

history was uneventful. He was pale and perspiring, the pulse rate was 92/min and regular blood pressure was 130/80 mm Hg. Many descrete small lymph nodes were palpated on his neck, axillae and groins. The liver was palpated 8 cm and spleen 10 cm below the costal margin, both firm, smooth and slightly tender. Chest X-ray and ECG were normal. Hemoglobin 9.7 g%, hematocrit 30%, reticulocytes 1.5%. White blood cell (WBC) count 114 000 μl, 82% lymphoblasts, 15% lymphocytes and 3% polymorphonuclears. Platelets 45,000/μl. The coagulation tests are given in table I. The bone marrow aspiration biopsy showed marked infiltration with lymphoblasts.

Acute lymphoblastic leukemia was diagnosed and the patient was treated with weekly intravenous injections of 1.5 mg/m vincristine for 3 weeks and daily administration of 40 mg/m² prednisone for 6 weeks. Two weeks after treatment was started, the liver spleen and lymph nodes were not palpated. The WBCs were 8,400/μl with a normal differential count. Starting on the 4th week the treatment was continued with mercaptopurine 70 mg/m² for 3 weeks and daily doses of cytosine arabinoside 80 mg/m² i.v. for 5 days. During the 2 subsequent months the patient was in clinical remission. Just before the scheduled central nervous system prophylactic irradiation, the patient showed relapse. He felt weak, the liver was palpated 5 cm and the spleen 2 cm below the costal margin. The WBC count increased to 135,000/μl with 80% blasts. Hemoglobin 10.8 g%, hematocrit 30%, platelet count 66,000/μl. Glucose 150 mg%, serum glutamic-oxalic transaminase 100 U (normal 5–40 U), serum glutamic pyruvic transaminase 78–123 U (normal 5–35 U), prothrombin time 30%. The abnormal liver function tests were interpreted as caused either by leukemic infiltration or by the mercaptopurine. The patient developed a severe myopathy

probably due to vincristine. Therefore, the treatment was continued by daily administration of prednisone 40 mg/m, mercaptopurine 3 mg/kg and intravenous methotrexate 20 mg/m² for 4 weeks. The WBC count decreased gradually reaching on the 9th day of treatment nadir of 3,000/µl with 49% blasts. On the 4th day of reinduction, epistaxis and a *few* petechiae on the legs appeared. The hemostatic tests (table I, April 8, 1975) were compatible with primary fibrinolysis. The patient was treated with ε-aminocaproic acid (EACA) 4 g every 6 h. The epistaxis stopped and the fibrinolysis subsided 3 days later, the coagulation tests returned to normal (table I, April 11, 1975). The patient was followed up and treated in the outpatient clinic with methotrexate 20 mg/m² twice weekly and prednisone 40 mg/m² daily. The WBC was 7,000/µl with a normal differential count, hemoglobin 10.5 g%, platelets 53,000/µl.

One month later he was admitted again because of repeated epistaxis and left shoulder pain of 3 days' duration. He was pale, cold and perspiring. Ecchymoses and petechiae covered his body. The pulse rate was 120/min, regular and the blood pressure 110/70 mm Hg. The spleen and the liver were enlarged 5 cm below the costal margin. The abdomen was distended and the left upper quadrant was tender. Shortly after admission the patient collapsed, the systolic blood pressure dropped to 80 mm Hg. *Hemoglobin 8.5 g%, WBC count 50,500/µl with 80% lymphoblasts. The* hemostatic tests showed marked fibrinolysis (table I, May 20, 1975). Ruptured spleen, relapse of the leukemia and fibrinolysis were diagnosed and the patient was operated on. The abdomen contained 1.5 liter of liquid blood without any clots. The spleen was very large, a bleeding tear at the inferior pole as well as signs of perisplenitis were seen. The liver was enlarged and pale-yellow. Splenectomy was performed. During the operation the patient received 7 U of whole blood and his blood pressure stabilized at 120/80 mm Hg. 4g EACA I. was given every 4 h. The hemostatic tests, though slightly improved, remained abnormal (table I, May 21, 1975). A few hours after the operation the patient's blood pressure suddenly dropped and he died. Postmortem examination was denied by the family.

The examination of the spleen showed: weight 1,060 g, few tears were seen on its lower pole. Light microscopy revealed destroyed structure and severe lymphoblastic infiltration.

Discussion

The patient's course was complicated by hemorrhagic episodes caused by fibrinolysis. The first episode was expressed by epistaxis and the appearance of petechiae. The coagulation tests showed thrombocytopenia, hypofibrinogenemia, prolonged prothrombin time, partial thromboplastin and thrombin time. The euglobulin lysis time and whole blood clot lysis time were both markedly shortened and the fibrinogen degradation products in the serum were elevated. We have disregarded the thrombocytopenia which was present and asymptomatic since the admission and was attributed to the leukemic process. The assumption of primary fibrinolysis

Table I. Results of the coagulation studies

	Normal values	Patient's values				
		10.12.74	8.4.75	11.4.75	20.5.75	21.5.75
Platelets/μl	150,000–400,000	45,000	40,000	35,000	27,000	48,000
Fibrinogen, mg%	200–400	250	110	160	80	260
Prothrombin time, %	100	80	30	60	2.	20
Partial thromboplastin time, sec	30–35	31	48	35	75	65
Thrombin time, sec	9–13	10	30	8	70	13
Euglobin lysis time, min	>120	140	30	130	20	30
Whole blood lysis time, h	>24	>24	1.5	>24	0.5	2
Fibrinogen degradation products, μg/ml	<10	10	>40	>10	>10	>10

history was uneventful. He was pale and perspiring, the pulse rate was 92/min and regular blood pressure was 130/80 mm Hg. Many descrete small lymph nodes were palpated on his neck, axillae and groins. The liver was palpated 8 cm and spleen 10 cm below the costal margin, both firm, smooth and slightly tender. Chest X-ray and ECG were normal. Hemoglobin 9.7 g%, hematocrit 30%, reticulocytes 1.5%. White blood cell (WBC) count 114,000 μl, 82% lymphoblasts, 15% lymphocytes and 3% polymorphonuclears. Platelets 45,000/μl. The coagulation tests are given in table I. The bone marrow aspiration biopsy showed marked infiltration with lymphoblasts.

Acute lymphoblastic leukemia was diagnosed and the patient was treated with weekly intravenous injections of 1.5 mg/m² vincristine for 3 weeks and daily administration of 40 mg/m prednisone for 6 weeks. Two weeks after treatment was started, the liver spleen and lymph nodes were not palpated. The WBCs were 8,400/μl with a normal differential count. Starting on the 4th week the treatment was continued with mercaptopurine 70 mg/m² for 3 weeks and daily doses of cytosine arabinoside 80 mg/m Lv for 5 days. During the 2 subsequent months the patient was in clinical remission. Just before the scheduled central nervous system prophylactic ir radiation, the patient showed relapse. He felt weak, the liver was palpated 5 cm and the spleen 2 cm below the costal margin. The WBC count increased to 135,000/μl with 80% blasts. Hemoglobin 10.8 g%, hematocrit 30%, platelet count 66,000/μl. Glucose 150 mg%, serum glutamic-oxalic transaminase 100 U (normal 5–40 U), serum glutamic pyruvic transaminase 78–123 U (normal 5–35 U), prothrombin time 30%. The abnormal liver function tests were interpreted as caused either by leukemic infiltration or by the mercaptopurine. The patient developed a severe myopathy

The primary fibrinolysis most probably was an additional cause for the spontaneous rupture of the spleen and the fatal outcome.

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based on the shortened euglobulin and whole clot lysis times which are usually normal in DIC and shortened in primary fibrinolysis [14]. The diagnosis was supported by the successful treatment with EACA. The second hemorrhagic episode was more fulminant, however the coagulation tests were similar and administration of EACA did correct some of the coagulation tests.

The first hemorrhagic episode occurred during a rapid lysis of large masses of leukemic cells, induced by chemotherapy. The second episode occurred during a rapid increase in the leukemic cell mass which was probably associated with a lysis of a great number of cells. CATTAN *et al.* [2] described two cases of acute monoblastic leukemia in which the antileukemic chemotherapy caused hemorrhage due to fibrinolysis. They also found increased fibrinolytic activity in the leukemic cells. It is possible that the leukemic cells of the presented patient released a plasminogen activator that caused the fibrinolysis. A probable contributing factor to the fibrinolysis was liver damage caused by the mercaptopurine, methotrexate and the hepatic leukemic infiltrate. The patient's course was further complicated by the spontaneous rupture of the spleen which eventually caused his death.

Primary fibrinolysis with hemorrhage is rare in patients with leukemia, but there are a few reports of such cases [1, 7, 8, 10]. OGSTON *et al.* [9] found increased fibrinolytic activity in 14 out of 43 patients with chronic lymphocytic leukemia, chronic myelocytic leukemia, acute lymphoblastic leukemia, acute myeloblastic leukemia and acute monoblastic leukemia. In a series of 110 patients with acute leukemia, KOHB [5] found shortened euglobulin lysis time in 70% of patients with parablastic acute granulocytic leukemia, in 50% with monocytic leukemia, in 31% with acute granulocytic leukemia and in 23% of patients with acute lymphoblastic leukemia.

Spontaneous rupture of the spleen is a rare complication of acute leukemia. RAVICH *et al.* [11] reviewed the literature in 1971 and found only 14 such cases. Since then there were only a few additional case reports [4, 6, 13]. Spontaneous rupture of the spleen in acute leukemia may be caused by rapid enlargement of the spleen due to leukemic infiltration resulting in increased intrasplenic pressure, by anoxia and infarction of the spleen, by coagulation abnormalities [3] and by rupture of enlarged capsular lymphatic vessel [12]. In the reported patient, the relapse of the disease, accompanied by a rapid increase in the leukemic cell mass, caused splenic infiltration, sudden enlargement of spleen and splenic rupture.

über 1000 abgestufte, aufeinanderfolgende Vergrösserungen desselben Feldes von lichtmikroskopischen bis zu niedrigen elektronenmikroskopischen Massen dargestellt. Diese zeigen sehr scharf und genau die wichtigsten Elemente und den Übergang zu komplizierten und feineren Strukturen. Auf der linken Seite befindet sich ein Text mit Beschreibung der Abbildungen und der morphologischen Details. Die Begriffe und Definitionen sind kurz, klar und prägnant. Die lichtmikroskopischen Präparate stammen von Menschen und Affen, die elektronenmikroskopischen von Ratten und Katzen. Die Reihenfolge der Organe und Gruppen ist eine Besonderheit, wie auch die Gruppierung von Zellen, Geweben, Systemen, Organen und Apparaten. Der letztere Begriff wird leider auch von diesem Autor nicht benutzt; so sind typische Apparate unter Systemen eingefügt (wie würde etwa die Bezeichnung «Radiosystem» oder «Ruder-» bzw. «Röntgensystem» klingen?) Es werden 36 Gruppen bzw. Kapitel mit ausführlichen Literaturangaben gebildet. Eine Hälfte des Werkes ist der Histologie, die andere Hälfte der makroskopischen Anatomie gewidmet. Das Kapitel über menschliche Blutzellen und Hämopoese ist unseres Erachtens die erste solide und klare Zusammenstellung der drei Blutzellarten. Man orientiert sich schnell und exakt (fette Buchstaben und Titel zu Beginn jeder Seite); so besteht eine fast ideale Kombination von Text, Beschreibung und Abbildungen, d.h. eines Lehrbuches und eines Atlasses, die das Studium sehr erleichtert. Anstelle von farbigen Darstellungen finden sich schwarz-weisse Abbildungen mit dem pädagogischen Zweck, die Vorstellungen von morphologischen und funktionellen Details neutral zu erfassen und anschliessend im Mikroskop mit verschiedenen in Anatomie und Pathologie verbreiteten Färbemethoden zu vergleichen. Das Werk weist eine gute didaktische, praktisch anwendbare, technische und wissenschaftliche Bearbeitung, Klarheit und Objektivität und eine hohe graphische Qualität auf und kann deshalb allgemein empfohlen werden.

Z. J. Dolduar, *Basel*

F. Mandelli, S. Amadori und G. Mariani: **Therapy of Acute Leukemias.** Minerva Medica, Roma 1975. 875 pp.

Dieses Buch gibt einen guten Überblick über den heutigen Stand der Therapie akuter Leukämien. Besonders hervorzuheben ist seine Vielseitigkeit. Nebst chemotherapeutischen werden immunotherapeutische Studien präsentiert. Als Nachschlagewerk ist es besonders wertvoll für seltene Leukämieformen, wo keine kooperativen internationalen Studien bestehen. Ebenfalls gut brauchbare Hinweise für die Behandlung von therapieresistenten Leukämien sind darin enthalten. Dieses Werk ist für hämatologisch-onkologisch tätige Ärzte sehr zu empfehlen.

B. Speck, *Basel*

Book Reviews Buchbesprechungen Livres nouveaux

L. Raw: **Anemia, from Molecules to Medicine.** Little, Brown, Boston 1975. XIII+ 264 pp., US$ 10.95

This book is the first of a proposed series of texts intended to foster student motivation for self study in the basic sciences by demonstrating immediate relevance to clinical medicine. Dysfunction is exemplified by anemia which is used to relate (red) cell structure to (red) cell function and thereby to cell dysfunction and to understanding of the disease state. Each chapter includes a study guide followed by a basic laboratory exercise to highlight the text which closes with a self evaluation section. Illustrations are helpful and ample.

A. Sawitsky *New Hyde Park, N Y*

J. Favre-Gilly, J. P. Pradier, J. P. Thouverez et J. Belleville: **Le facteur stabilisant de la fibrine (FSF) et son déficit congénital.** Imprimerie des Beaux Arts, Lyon 1974. 260 pp., 46 tab., 12 fig.

Cette monographie de 223 pages sur le facteur stabilisant de la fibrine nous donne un bon aperçu du facteur XIII. Il contient une liste presque complète de tous les cas publiés jusqu'en 1974 avec une description détaillée de chacun d'entre eux qui tient compte des examens de laboratoire, des symptomes cliniques et de la famille. Un chapitre est réservé à l'étude clinique analytique générale de tous les cas. On y retrouve toutes les formes d'hémorragie, leur évolution, leur fréquence. Une table récapitulatrice aurait peut-être donné une vue générale encore plus précise et plus facile à assimiler que la simple énumération. Le chapitre réservé aux propriétés biochimiques du facteur XIII s'en tient aux grands traits, les découvertes les plus récentes n'ont malheureusement plus pu être considérées. L'analyse génétique inclut divers aspects fort intéressants du facteur XIII. Le dernier chapitre me semble important, il résume les points essentiels du diagnostic, du pronostic et du traitement en rappelant que le traitement prophylactique par la durée de son action est particulièrement indiqué pour les malades déficients en facteur XIII. Cette monographie est intéressante par la somme des informations qu'elle donne.

F. Duckert *Bâle*

J. A. G. Rhodin: **Histology. A Text and Atlas.** Oxford University Press, New York 1974. 803 pp., ca. 1,100 fig., £ 9.00.

Das Buch wurde für Studierende und Spezialisten der Biologie bzw. Human- und Veterinärmedizin geschrieben. Der Aufbau erfolgte in einer neuen, nicht ganz unbekannten Form und Verteilung der Materie. Auf der rechten Seite sind jeweils

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Bearbeitet von G. B[illegible], Basel

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International Society for Experimental Hematology

The *6th Annual Conference of the ISEH* will be held at Basel, Sandoz Ltd Convention Centre, from August 28 to 31, 1977 chairman. BRUNO SPECK, MD, Basel.

Invited plenary sessions and free papers on *Hemopoietic stem cells*. regulation, differentiation the hemopoietic inductive microenvironment exogenous effects on hemopoiesis, experimental models of hematologic disorders. *Immunity* lymphocytes, monocytes, macrophages; histocompatibility testing· cell interactions; immune suppression and stimulation immune deficiency states, immunotherapy· cellular engineering· clinical and experimental neoplasias of hemopoietic tissues. *Bone marrow transplantation*. experimental models, clinical marrow grafts for immune deficiency syndromes, aplastic anemia, malignant disease and other indications, grafts across MHC and ABO barriers.

Annual Meeting of the International Cooperative Group for Bone Marrow Transplantation in Man. September 1, 1977

Congress language English. No simultaneous translation is provided.

All correspondence should be sent to Congress Secretariat ISEH, PO box 129 *CH-4004 Basel* Switzerland.

1st Florence Conference on Haemostasis and Thrombosis

Florence May 10–12 1977

Scientific Committee B A. KUDRJASHOV (URSS), M J LARRIEU (France), G. G. NERI SERNERI (Italy) I. M. NILSSON (Sweden), M. VERSTRAETE (Belgium) chairman: G G NERI SERNERI.

Themes. Immunology and biochemistry of fibrinogen and factor VIII. Oral contraceptives and thrombosis. Platelet aggregation, inhibition and immunology Vessel wall, fibrinolysis and thrombosis. Relationship between coagulation and other systems. Neurohumoral control of haemostasis.

Official languages: English and Italian (simultaneous translation).

Organizing and scientific secretariat Dr G. G NERI SERNERI, Istituto di Patologia Medica II dell'Università di Firenze, Viale Morgagni 85, *Firenze* Italy

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